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Phytopathology

An International Journal

Official Organ of
The American Phytopathological Society

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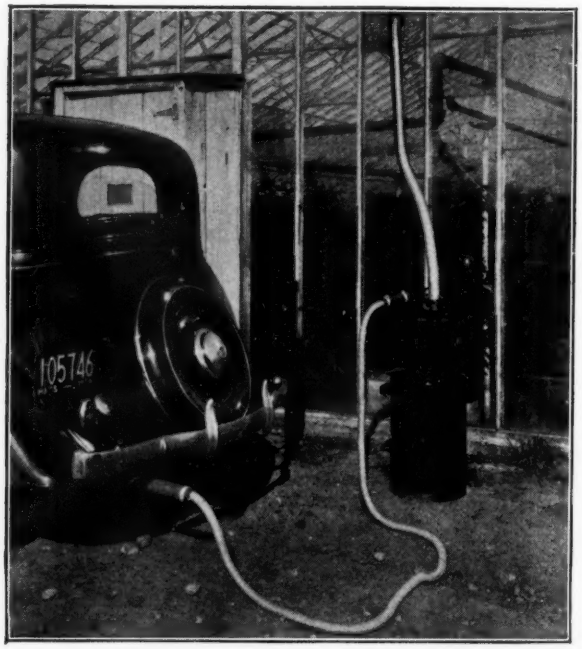

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PHYTOPATHOLOGY

VOLUME 24 NUMBER 8

AUGUST, 1934

A MASKED STRAIN OF TOBACCO-MOSAIC VIRUS[1]

FRANCIS O. HOLMES

(Accepted for publication April 24, 1934)[2]

INTRODUCTION

A number of mild-mottling, attenuated strains[3] of tobacco-mosaic virus have been described by J. Johnson (5, 6), Johnson and Grant (7), and E. M. Johnson (4). Tobacco plants infected with these attenuated strains show less distortion and less conspicuous mottling than appear in those infected with the common, or field-type, tobacco-mosaic virus. Attenuated strains have been reported to vary among themselves (5), some consistently producing milder symptoms than others; but no strain of tobacco-mosaic virus has been described as symptomless in *Nicotiana tabacum* L.

In the course of experiments on rate of increase of tobacco-mosaic virus at high temperature, the writer isolated a strain that was able to infect, increase in, and spread systemically in *Nicotiana tabacum* var. Turkish and certain other plants, without producing appreciable mottling, blanching, distortion, or stunting. In spite of the masked character of its infection in *N. tabacum*, this strain of virus showed many of the fundamental characteristics of the common field strain, and was indistinguishable from it in some hosts. In large sets of tobacco plants inoculated with the masked strain, a few individual plants showed transient and indistinct traces of clearing of veins and of obscure mottling. It was generally not possible, however, to distinguish Turkish tobacco plants infected with this strain of tobacco-mosaic virus from noninfected, healthy plants of the same age. The presence of the masked-strain virus in infected plants was occasionally betrayed by the appearance of isolated yellow spots, which were found to be sources

[1] This masked strain of tobacco-mosaic virus was isolated and its properties were studied during 1930, 1931, and 1932 at the Boyce Thompson Institute for Plant Research, Yonkers, N. Y. Repurification of the original tobacco-mosaic stock virus and experiments on the origin of attenuated strains were performed in 1932 and 1933 at The Rockefeller Institute for Medical Research, Princeton, N. J.

[2] Published at the expense of The Rockefeller Institute for Medical Research out of the order determined by date of receipt of the manuscript.

[3] The results of the work reported in the present paper give evidence that these attenuated forms of tobacco-mosaic virus merit the designation "strains." For convenience, this term is used throughout.

of yellow-mosaic virus strains more or less similar to the strains arising in plants infected with ordinary tobacco-mosaic virus, as described by McKinney (9) and Jensen (3).

It is the purpose of the present paper to report experiments in which this masked strain and other attenuated strains were isolated, and to describe the properties of the masked-strain virus.

ORIGIN OF MASKED STRAIN

The masked strain of tobacco-mosaic virus was originally obtained in the course of the following experiment. Pieces of healthy tomato stem were inoculated with tobacco-mosaic virus of a stock isolated from a single necrotic lesion on *Nicotiana glutinosa* L. and previously used in studies of symptoms induced in a variety of host plants (2). The pieces of stem were incubated in loosely plugged, sterilized test tubes in a thermostatically controlled water-bath at 34.6° C. After 15 days' incubation, several stems were crushed and the expressed juices from each were used to inoculate plants of *N. glutinosa.* The appearance of numerous necrotic lesions on a single plant indicated that considerable virus had been produced in one of the stem pieces, but not in the others. Transfers were made from 10 of the lesions to 10 young Turkish tobacco plants. The transfer of virus from a necrotic lesion in *N. glutinosa* was made by grasping part of an infected leaf between two wooden pot labels, tearing away a bit of leaf tissue that included a necrotic lesion, crushing the tissue between the sticks, and rubbing the expressed fluid over the surface of a leaf of *N. tabacum.* This process had been found to infect at least one-half of all inoculated plants in past experiments. In this experiment, however, 9 of the 10 inoculated plants showed no symptoms as a result of the inoculation. The 10th plant showed a type of mottling milder than any previously observed. The presence of this extremely mild mottling in one plant suggested that a test be made for possible virus content of the 9 symptomless plants. A transfer was made from each of the symptomless plants to a plant of *N. glutinosa.* Numerous necrotic lesions appeared on three plants, the other 6 transfers causing no symptoms. It, therefore, appeared that a masked infection with a strain of tobacco-mosaic virus had been present in 3 of the Turkish tobacco plants. From the necrotic lesions on *N. glutinosa,* the virus was again transferred to healthy Turkish tobacco plants, and was maintained for a time by serial transfers in that variety of *N. tabacum.* No symptoms were seen in the living plants, although evidence of interference with carbohydrate metabolism in recently infected tissues was found upon staining leaves with iodine. A stock of this masked virus was then derived by transfer from an isolated single lesion on *N. glutinosa* to Turkish tobacco and later to Bonny Best tomato, the juice of which was diluted 1 : 20 in water and preserved by freez-

ing. The strain of virus represented by this frozen stock will be referred to in this paper as the single-lesion masked strain of tobacco-mosaic virus.

For comparison with the masked strain, and for producing mixtures of known constituents, two other frozen stocks of virus were available. The first was the stock from which the masked strain had been derived; it represented a previously-described (2) single-lesion strain of the ordinary or field-type of tobacco-mosaic virus, hereafter referred to as the distorting strain. The second was a single-lesion stock of an attenuated strain isolated from the distorting-type stock by growth at 34°–35° C., characterized by mottling, but no distortion on *Nicotiana tabacum*, and hereafter referred to

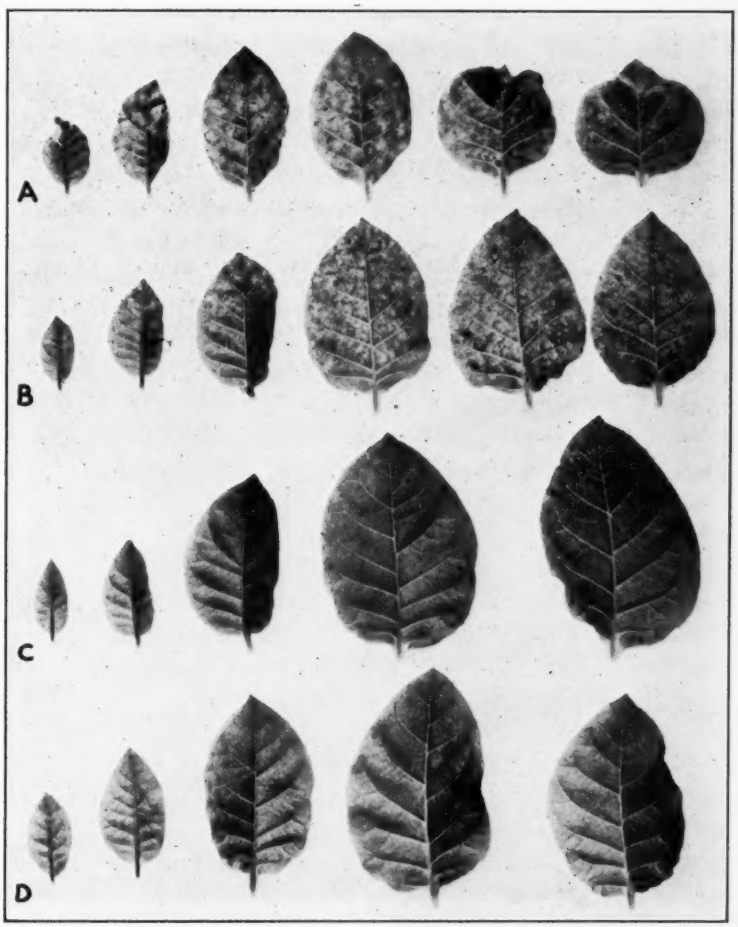

FIG. 1. Successively larger leaves from tops of Turkish tobacco plants 26 days after infection with three strains of tobacco mosaic. A. Distorting. B. Mottling. C. Masked. D. Corresponding leaves of healthy plant of same age.

as the mottling strain. Leaves from the tops of three plants infected with distorting, mottling, and masked strains of tobacco-mosaic virus, and corresponding leaves of a noninfected plant are shown in figure 1.

PROPERTIES OF MASKED STRAIN

Host Range; Symptoms

The masked strain of virus was used to inoculate a number of species and varieties of plants in order that the resulting infections might be compared with infections by the distorting and mottling strains in the same hosts.

Six species that mottle when infected with the distorting strain were inoculated; they were *Nicotiana tabacum* varieties Turkish, Burley, Connecticut Seed Leaf, Green's Wildfire-Resistant Orinoco, and *chinese*, *N. palmeri* A. Gray, *N. longiflora* Cav., *N. sauveolens* Lehm., *N. sylvestris* Spegaz. and Comes, and *N. trigonophylla* Dun. These six species showed no symptoms with the masked strain, unless a few whitened small veins in some plants of *N. sauveolens* were such; they showed yellowish primary lesions, puckering of young leaves, clearing of veins, and subsequent mottling and distortion with the distorting strain of virus, and yellowish primary lesions, slight clearing of veins, and comparatively early mottling without distortion with the mottling strain. *Solanum nigrum* L. var. *nodiflorum*, which mottles obscurely when infected with the distorting strain, also showed no symptoms when infected with the masked strain.

Three species that respond by mottling preceded by marked stunting and blanching, or by mottling and formation of leafy-outgrowths, or enations, when inoculated with the distorting strain, were tested. The first two of these, *Nicotiana clevelandii* A. Gray, and *N. bigelovii* Wats. varieties *quadrivalvis* Pursh. and *multivalvis* Lindl., which show prolonged stunting and extensive blanching of young leaves when infected with the distorting strain, also showed a tendency toward pronounced symptoms by responding with some distortion to the mottling strain, which does not cause distortion in *N. tabacum*, and with faint but distinct mottling to the masked strain, which produced no obvious symptoms in simultaneous tests in *N. tabacum*. The third species, *N. paniculata* L., which shows enations on the lower sides of severely distorted leaves when inoculated with the distorting strain of virus, demonstrated this tendency to show severe symptoms by a little distortion of leaves in plants infected with the mottling strain of virus and a slight unevenness of color in leaves of plants infected with the masked strain; no enations were caused by the mottling or masked strains.

In all of the above-mentioned host plants, the degree of stunting was correlated with the intensity of chlorosis in the mottling pattern. In gen-

eral, the distorting strain caused relatively severe stunting for a considerable period after infection; the mottling strain caused moderate stunting; and the masked strain caused so little stunting that plants infected with it could not readily be distinguished in this respect from healthy control plants.

Systemic necrosis caused by the three virus strains was studied in *Nicotiana rustica* L. In the relatively young plant, just beginning to form an elongated stem after the rosette stage, the distorting strain caused necrotic primary lesions, severe stunting, puckering of youngest leaves, clearing of veins, early necrosis of small veins in the same area, subsequent death of young leaves and parts of the stem, and finally death of the whole plant. The mottling strain caused similar necrotic primary lesions, but a milder systemic disease with but slight stunting; many necrotic lesions appeared in partly grown leaves, causing leaf distortion, but the plants did not die. The masked strain induced the appearance of similar necrotic primary lesions, but no noticeable stunting; only a few isolated necrotic lesions appeared in partly grown leaves, and these caused little or no disturbance to growth of the infected plant. It appears that the masked strain of virus did not invade the noninoculated part of *N. rustica* nearly so extensively as did the other strains. Symptoms of infections by the three strains could be distinguished from one another in the systemic phase of the disease, but not by the primary lesions, which were approximately equal in number and indistinguishable in size, color, and general appearance.

A number of species in which infection commonly does not become systemic, but that show only necrotic primary lesions as a result of inoculation with the distorting strain, were tested by inoculation with the masked and the mottling strains. These were *Nicotiana langsdorffii* Schrank, *N. acuminata* Hook., *N. glutinosa*, *N. sanderae* Sander, *N. alata* Link and Otto, the F_1 hybrids *N. tabacum* × *glutinosa*, *N. paniculata* × *rustica*, and *N. glauca* R. Grah. × *langsdorffii*, *Capsicum frutescens* L. (necrotic type), *Physalis angulata* L., *Solanum pseudo-capsicum* L., *Datura stramonium* L., and several varieties of *Phaseolus vulgaris* L. In all cases, the necrotic lesions of the three strains appeared alike on any given host species or variety. Necrotic lesions representing the three strains of virus are shown in figure 2 as they appeared on inoculated leaves of *N. langsdorffii*.

One host species that is capable of acting as a symptomless carrier of tobacco-mosaic virus of the distorting strain was tested with the masked strain. This species, *Nicotiana glauca*, always shows mild mottling if infected when very young with the distorting strain; this mottling is often outgrown, with the result that many plants finally become symptomless, although containing virus in some leaves. *N. glauca* showed no symptoms at any stage of growth when infected with the masked strain of virus,

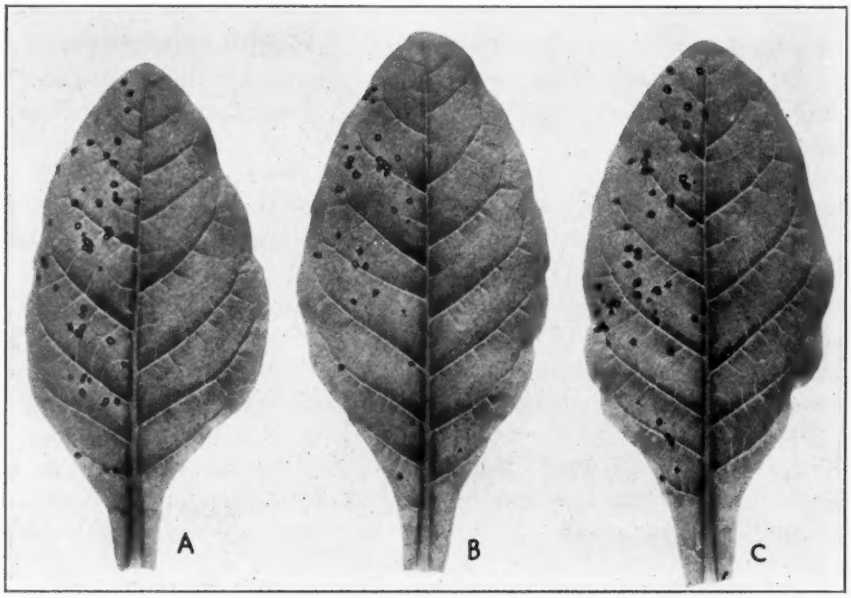

FIG. 2. Necrotic lesions on leaves of *Nicotiana langsdorffii*, inoculated with three strains of tobacco-mosaic virus. A. Masked. B. Mottling. C. Distorting.

although virus increased at the site of inoculation and spread to some extent to noninoculated leaves.

To summarize the above observations, it appears (1) that the host range of the three strains of tobacco-mosaic virus was coextensive, so far as tested, no host of one strain having been found immune from another. (2) Primary lesions of necrotic type were indistinguishable in infections by the three strains. Yellowish primary lesions were shown in some hosts by the distorting and mottling strains, but not by the masked strain. (3) All systemic infections with the three strains showed the same sorts of differences. The distorting strain was consistently associated with the most severe stunting, mottling, and distorting, or with the most extensive necrosis; the masked strain induced the least effect; and the mottling strain was associated with symptoms of intermediate type.

Starch Retention in Leaves

There were changes in invaded tissues of *Nicotiana tabacum*, in spite of the absence of obvious symptoms. These were changes in carbohydrate metabolism, resulting in starch-retention patterns similar to those previously demonstrated in plants infected with distorting-strain virus (1). By incubation of infected plants for about 16 hours at 10° C. and 4 to 6

hours at 22° C. in darkness, after full sunlight exposure, and by subsequent treatment of leaves with 95 per cent ethyl alcohol to remove chlorophyll, then with 0.6 per cent iodine in 2 per cent potassium iodide solution to stain starch where present, starch-retention patterns were made visible. It was thus possible to show that the masked-strain virus first involved small circular areas in the inoculated leaf. It then affected tissues along main veins and entered such leaves at the top of the plant as would have been affected by clearing of veins in the living plant if distorting-strain virus had been used. The starch-retention patterns at the clearing-of-veins stage were similar in masked and distorting-type infections. In later stages of the disease, however, the masked strain did not appear to invade as extensive areas as were involved by the other strains. The younger leaves of plants infected with the masked strain showed only a few centers of infection in these later stages, as judged by starch-retention lesions. These scattered centers of secondary infection were marked by discrete, ring-like lesions, which were smaller in younger than in older leaves and in basal parts of individual leaves than in apical parts of the same leaves. Figure 3, A, represents leaves from the top of a single plant infected with virus of the masked strain; comparatively few, small lesions of the systemic infection appear in the youngest leaves and younger parts of older leaves. The mottling strain produced more centers of infection in young leaves, but a similar gradient in size of lesions with increasing maturity of leaf tissue. This is represented in figure 3, B, in which leaves from the top of a plant inoculated 12 days earlier show secondary lesions of varying size, in contrast to figure 3, C, in which are shown primary lesions, all of approximately equal size, on similar leaves of a control plant inoculated over all available leaf surface three days before treatment by the iodine-staining method. The fact that lesions develop to approximately the same size in all parts of young leaves inoculated at the same time, but to different sizes in similar parts of leaves infected systemically, suggests that systemic infection of these tissues occurs progressively as they reach a definite degree of maturity. The distorting strain, upon entering young leaves, sets up so many centers of infection in them that separate points of secondary infection could seldom be discerned.

Plants of *Nicotiana glauca* were inoculated with the masked and distorting strains of virus, and their leaves were later stained by the iodine method for demonstrating recently invaded areas of tissue in the systemic infection. The starch-retention lesions in noninoculated, but systemically infected, leaves of young plants of *N. glauca* infected with distorting-strain virus were few. Such as did occur were discrete and ring-like, resembling the starch-retention lesions of the masked strain in *N. tabacum*. In older, symptomless plants of *N. glauca* infected with the distorting strain, the

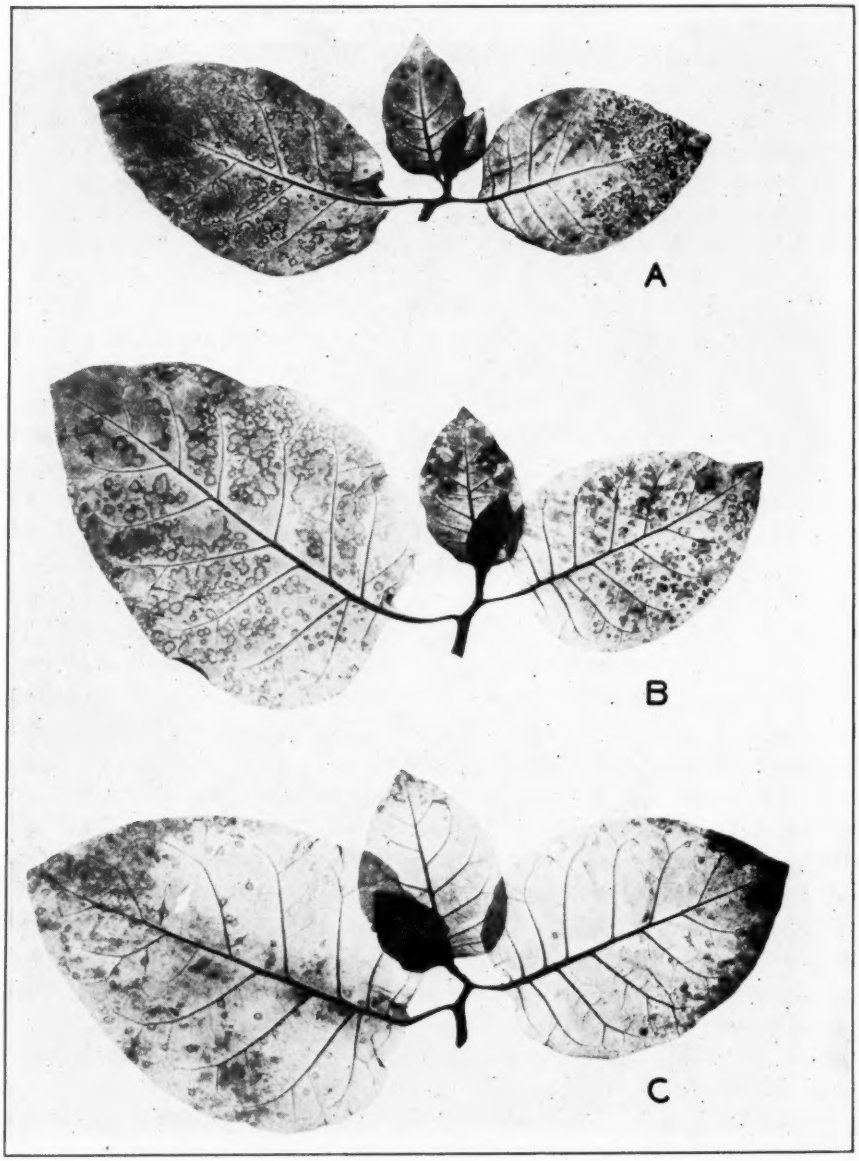

FIG. 3. Leaves stained with iodine to show starch-retention patterns. A. Top of plant systemically infected with masked strain of tobacco-mosaic virus. B. Top of plant systemically infected with mottling strain and showing conspicuous gradient in size of secondary lesions. C. Top of control plant, 3 days after inoculation over whole leaf surface, with mottling strain, showing primary lesions all of approximately equal size.

upper leaves showed even fewer starch-retention lesions. In *N. glauca* plants infected with masked-strain virus, no symptoms were seen in the living plants. After staining leaves with iodine, primary lesions were found to be as numerous as those in infections with distorting-strain virus, but lesions in noninoculated leaves, representing the systemic phase of the disease, were few in number. In some plants no systemic infection occurred.

Failure of extensive invasion of young leaves seems to be characteristic of the symptomless condition in hosts of tobacco-mosaic virus. This was demonstrated and described in an earlier paper (2) as a result of studies of the concentration of virus present in leaves of different ages in plants of the symptomless *Solanum melongena* var. Hangchow Long infected with distorting-strain virus. After publication, it was found that *S. sanitwongsei* Craib, another symptomless host of the distorting-strain virus, showed this failure of extensive invasion even more strikingly. In this plant, even long after infection, many leaves near the growing points of branches were almost, or entirely, free of virus; older leaves showed considerably greater concentrations of virus as a result of systemic infection, the oldest leaves being excellent sources of distorting-strain virus. Thus the virus appeared to lag behind the growth of the plant. The present demonstration by a visual method, that is, by staining systemically infected leaves, suggests inclusion of the masked-strain infection of *Nicotiana tabacum* in the same category as infections in symptomless hosts by the distorting strain of tobacco-mosaic virus.

It seems possible that this failure of the masked strain of virus to move freely into young tissues of the plant or to increase much there, may explain in part the symptomless character of the infection. When tissues are finally invaded they may be old enough to be little harmed by the presence of the virus. On the other hand, the fact that primary lesions are not visible on leaves of *Nicotiana tabacum* inoculated with virus of the masked strain, as they are on similar leaves inoculated with the distorting strain, and the fact that the area of clearing of veins, invaded similarly by both strains, is not outwardly affected by the masked strain, indicate also that the presence of virus of the masked strain is less injurious even in invaded tissues.

Increase at High Temperature

Preliminary tests showed that the masked strain of tobacco-mosaic virus was able to increase at a temperature slightly higher than the highest temperature previously found suitable for increase of the distorting strain. This was confirmed by simultaneous tests as indicated in the following experiment.

Stems of healthy Bonny Best tomato plants, cut into 3-inch lengths, were divided into three equal sets and inoculated with the distorting, mottling, and masked strains, respectively. Each piece of stem, after inoculation, was dropped into a test tube. The three lots of tubes were then immersed as soon as possible in a thermostatically regulated water-bath and maintained at a temperature of 35.45 ± 0.03° C. Tests of concentration of virus in 2 pieces of stem from each lot were made at intervals, using the number of lesions developing on *Nicotiana glutinosa* plants inoculated with juices from the crushed stems as a measure of concentration. The results of the experiment are shown in table 1. The mottling and masked strains of virus produced increasing numbers of lesions in successive tests, but the distorting strain did not. The result of this experiment suggests that the distorting

TABLE 1.—*Comparison of ability of three strains of tobacco-mosaic virus to multiply in pieces of stem incubated at 35.45 ± 0.03° C.*

Interval between stem inoculation and test	Distorting strain			Mottling strain			Masked strain		
	1st test	2nd test	Average	1st test	2nd test	Average	1st test	2nd test	Average
1 day	1[a]	0	0.5	1	0	0.5	10	2	6
2 days	0	0	0	6	0	3	25	17	21
3 days	0	0	0	0	4	2	43	61	52
5 days	1	1	1	61	30	46	165	42	104
7 days	1	0	0.5	152	180	166	180	158	169
10 days	0	0	0	160	285	222	397	717	557

[a] Figures represent numbers of lesions produced on 5 leaves of *Nicotiana glutinosa* as a result of inoculation with sample indicated.

strain is unable to increase at a temperature at which the attenuated strains increase rapidly.

It might be, however, that at a high temperature the more injurious distorting strain would fail to become established, perhaps killing the first invaded cells or injuring them so as to prevent spread to the remainder of the tissues within the time of the experiment. Ability of the strains to grow at the elevated temperature might be equal if they were once established. To test the possibility that the distorting strain might multiply at high temperature, if once established in the tissues, an experiment was performed in which inoculated stems were not subjected to the higher temperature until 24 and 48 hours after inoculation. Stems of Turkish tobacco were tested for freedom from virus by inoculation of *Nicotiana glutinosa*

plants with juices from part of each. Subsequent failure of the *N. glutinosa* plants to show necrotic lesions was considered evidence that the selected stems were actually not infected before inoculation. The healthy stems were cut into 2-inch lengths, and each piece was inoculated by 30 pin punctures, using as inoculum a single-lesion stock of distorting-type virus, previously stored frozen at 1:20 dilution in water. The inoculated stem pieces were dropped into sterilized bacteriological test tubes. The stems were partly protected from drying by the addition of a short section of younger and noninoculated stem, and by plugging the tubes with cotton. The stems in their tubes were then divided at random into four lots, the first being subjected to a temperature of 21° C. at once, the second to 34.2° C. in a water-bath, the third to 21° C. for 24 hours and then to 34.2° C., and the fourth to 21° C. for 48 hours and then to 34.2° C. Virus increase during ten days was rapid at 21° C., as shown by frequent tests of virus concentration in samples of the stems; it was inhibited completely at 34.2° C. in the sample subjected to this temperature at once, and allowed in a few stems of the samples held for a time at the lower temperature, but finally transferred to 34.2° C. The increase was considerable, however, for only two individual pieces of stem, one incubated 24 hours at 21° C. and then 9 days at 34.2° C., the other incubated 48 hours at 21° C. and then 5 days at 34.2° C. From lesions produced on *N. glutinosa* by inoculation with juices of one of these stems, 34 isolations were made by transfer to young Turkish tobacco plants. All 34 proved to be attenuated strains. A majority of similar isolations from the second stem was also attenuated. The opportunity afforded the distorting-type virus to become established in the cells of the stem before being subjected to high-temperature treatment did not, therefore, allow the unchanged distorting-type virus to increase under these conditions. Later experiments of the same general sort confirmed this result and showed that when any considerable increase of virus occurred in stems subjected to such elevated temperature, even after the distorting-type virus was allowed 48 hours at room temperature to establish itself after inoculation, subsequent isolations on Turkish tobacco showed that the distorting-type virus had been partly or wholly suppressed and some attenuated strain had appeared. The distorting-type virus did not increase at such temperatures, even when an opportunity was given for the virus to become established in the tissues at a lower temperature. The ability to increase at the relatively elevated temperature may, therefore, be considered a differential characteristic of the masked strain of tobacco-mosaic virus described in this paper.

Response to Dilution

One of the most striking characteristics of tobacco-mosaic virus is the high infectivity shown when juices of infected plants are diluted with pro-

gressively increasing amounts of water and used as inoculum. Undiluted expressed juices of plants of *Nicotiana tabacum* infected with the masked strain of tobacco-mosaic virus appeared to be nearly as infectious as similar extracts from plants infected with the distorting strain. It was conceivable, however, that progressive dilution with water might bring out some striking difference. Samples were therefore prepared and tested.

Freshly expressed juices of Turkish tobacco plants, infected 25 days previously with masked and distorting strains of tobacco-mosaic virus, were used undiluted and in water dilutions to inoculate *Nicotiana glutinosa* plants. The results, as averages of the number of necrotic lesions on 4 plants at each dilution, were as follows: undiluted samples representing masked strain and distorting strain respectively, 2624 and 2965 lesions per plant (5 leaves); dilutions of 1:10 in water, 1768 and 1930 lesions; of 1:100, 1024 and 1223 lesions; of 1:1000, 499 and 824 lesions; of 1:10,000, 36 and 210 lesions; of 1:100,000, 6.2 and 32 lesions; of 1:1,000,000, 1.5 and 6.8 lesions.

The effect of dilution appears entirely similar for the two strains, both producing moderate numbers of infections when used as inoculum in 1:1,000,000 dilution. The measurements indicated that at all dilutions the masked-strain sample acted as though less concentrated than the distorting-strain sample. This has been observed in other experiments. The observation that iodine-stained leaves show a few scattered secondary lesions instead of numerous ones, such as are characteristic of distorting-strain infections, might explain a lower concentration of virus in expressed juices of plants serving as sources of inoculum.

Approximate Thermal Death Point

The masked strain was tested to determine whether its thermal death point was as high as that of the distorting strain, which is outstanding among viruses for its ability to remain infective after treatment at high temperatures. Samples of the masked, mottling, and distorting strains of virus, previously preserved frozen in 1:20 dilutions of juice of infected plants, were thawed and exposed for 10 minutes to temperatures ranging from 70° to 90° C. At each temperature the three strains of virus were exposed simultaneously, being immersed in narrow glass tubes together in a water-bath, the water being agitated by a stirring device. Each sample was tested, after heating, by using it to inoculate two *Nicotiana glutinosa* plants. The results, shown in table 2, indicate that all three samples were reduced to about the same extent by the temperatures 75° and 80° C., and that all failed to infect after treatment at 85° and 90° C. The thermal death point of the masked strain is, therefore, approximately the same as that of the other strains of tobacco-mosaic virus.

TABLE 2.—*Effect of 10-minute exposures of three strains of tobacco-mosaic virus to various temperatures in vitro*

Temperature	Distorting strain			Mottling strain			Masked strain		
	1st test	2nd test	Average	1st test	2nd test	Average	1st test	2nd test	Average
70° C.	148[a]	94	121	54	132	93	19	32	26
75° C.	13	2	8	41	22	32	6	3	4
80° C.	6	4	5	5	6	6	2	0	1
85° C.	0	0	0	0	0	0	0	0	0
90° C.	0	0	0	0	0	0	0	0	0
Unheated	177	148	162	118	146	132	24	82	53

[a] Figures represent numbers of lesions produced on 5 leaves of *Nicotiana glutinosa* as a result of inoculation with sample indicated.

Stability During Successive Transfers in Plants

The stability of the masked strain was tested by 10 successive transfers through sets of Turkish tobacco plants at greenhouse temperature, about 21° to 24° C. Observations were made of the occurrence of occasional aberrant symptoms and of the similarity of plants infected in the final transfer of masked-type virus and plants infected directly from the frozen stock used to initiate the series.

The first infection was obtained by inoculation of 20 plants with frozen stock of juice representing the masked strain. An equal or larger number of noninoculated plants were intermingled with the infected individuals of each set as a control to detect possible accidental infections and to afford healthy foliage for comparison with that of plants infected with the masked strain of virus. Each successive set of 20 or more plants was inoculated from a systemically infected, but not originally inoculated, leaf of one plant of the preceding set.

In the 10th set of the series, 50 infected plants were compared with 50 plants inoculated with the original frozen stock of masked-strain virus. The two sets of plants were kept under close observation. At the end of the first week, noninoculated leaves of 10 plants from each set were tested by inoculation of *Nicotiana glutinosa;* it was found that the masked virus was present in each in spite of the absence of visible symptoms. The infected plants of the 10th set showed no more symptoms than did the plants infected with masked-strain virus from the original frozen stock.

During the course of this experiment, the masked-strain virus was carried serially to 10 sets containing a total of 230 plants of *Nicotiana*

tabacum; 390 control plants were used. No symptoms were observed in any of the control plants. Among the 230 inoculated plants, 5 showed visible and hence atypical symptoms; these were mottling patterns, somewhat resembling the usual symptoms of the mottling strain. Fourteen plants showed one or more bright yellow spots; yellow-mosaic strains were isolated from some spots of this type. To this slight extent, at least, the strain was probably unstable. The fact that this strain was capable of being passed through 10 serial transfers at greenhouse temperature without losing its individuality indicates, however, that it is a reasonably stable type of tobacco-mosaic virus.

Infectivity After Long Storage

A test of the ability of the masked strain to remain infective after storage was made with dried leaf material and frozen diluted extract of infected plants. This was done to determine whether the masked strain resembled the distorting strain of tobacco-mosaic virus in its property of withstanding long storage. This property of ordinary tobacco-mosaic virus is not shared by certain other mosaic viruses, such as those of cucumber mosaic, and etch of tobacco (4). An example of rapid spontaneous inactivation has recently been described by Samuel and Bald (12, p. 96) who show that the virus of spotted wilt of tomato becomes entirely uninfective in expressed juice in less than 6 hours at 19° C. and in 3 hours at 22° C.

A fragment of dried leaf in which the masked strain of virus had been preserved from November 23, 1931, to October 13, 1933, was crushed in a sterile mortar with a little water on the latter date. The resulting suspension was used to inoculate plants of *Nicotiana tabacum* var. Turkish and *Datura stramonium*. The infections on *N. tabacum* proved to be typical for the strain, and the necrotic lesions on *D. stramonium* were indistinguishable in appearance and approximately as numerous as control lesions of the distorting strain produced by treating dried leaf material of that strain in a similar way. It is evident that the masked strain of tobacco-mosaic virus is to be considered capable of prolonged storage in dry leaf tissue without becoming inactive, the sample used in this experiment having been preserved for nearly two years before use.

A stock of masked-strain virus was tested after it had been kept frozen two years and ten months in a 1:20 dilution of expressed juice in water. It was found still highly infectious, giving approximately as many lesions on *Nicotiana glutinosa* plants as it had, at the same dilution, when freshly expressed. It produced typical masked infections in plants of *N. tabacum* var. Turkish.

These experiments gave evidence that the ability of ordinary tobacco-mosaic virus to withstand storage for long periods is shared by virus of the masked strain.

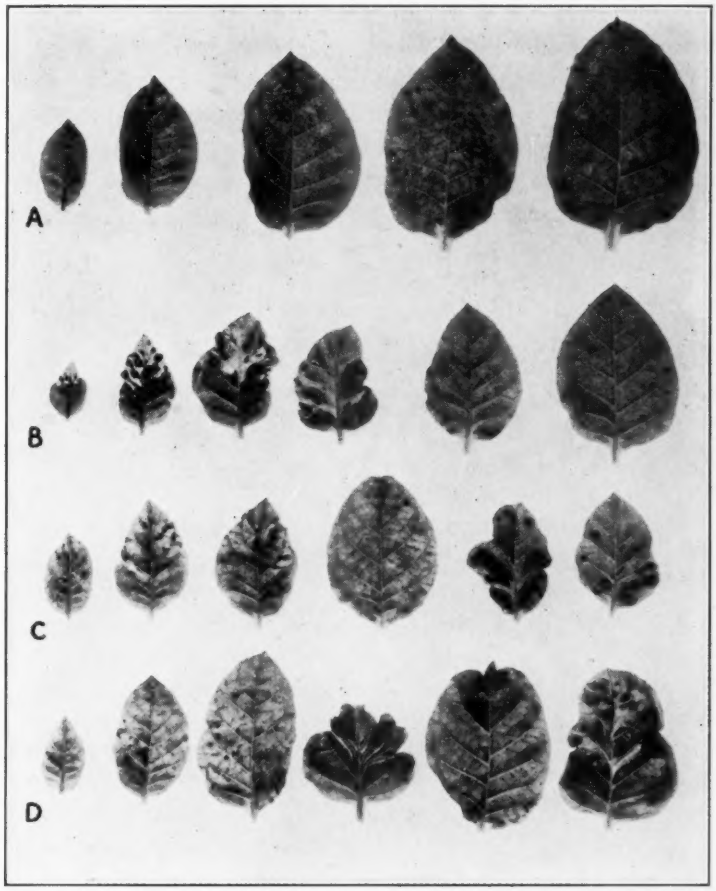

FIG. 4. Successively older leaves from tops of Turkish tobacco plants 26 days after infection with mixtures of strains of tobacco-mosaic virus. A. Masked and mottling strains. B. Masked and distorting strains. C. Mottling and distorting strains. D. Masked, mottling, and distorting strains.

Behavior in Mixtures with Other Strains

Mixture Patterns. Turkish tobacco plants inoculated with mixtures of strains showed approximately the symptoms characteristic of the more severe strain of the mixture. This is shown in figure 4 (compare with single-strain infections represented in Fig. 1). An additional peculiar symptom, spotting of a leaf originally showing clearing,[4] appeared, however, in many of the plants infected with mixtures of strains. The spotting

[4] The term "clearing" is hereinafter used to designate the symptom previously called "clearing of veins." It is suggested that the longer term is difficult to use in certain phrases, and that the word clearing will prove convenient and distinctive.

became evident after clearing, itself, had nearly disappeared; it was generally conspicuous and erratic in pattern, the spots being characteristically angular rather than rounded. This type of spotting was absent from infections with single-lesion strains of each type. Common forms of mixture patterns and normal clearing in leaves from distorting-strain and mottling-strain infections are represented in figure 5.

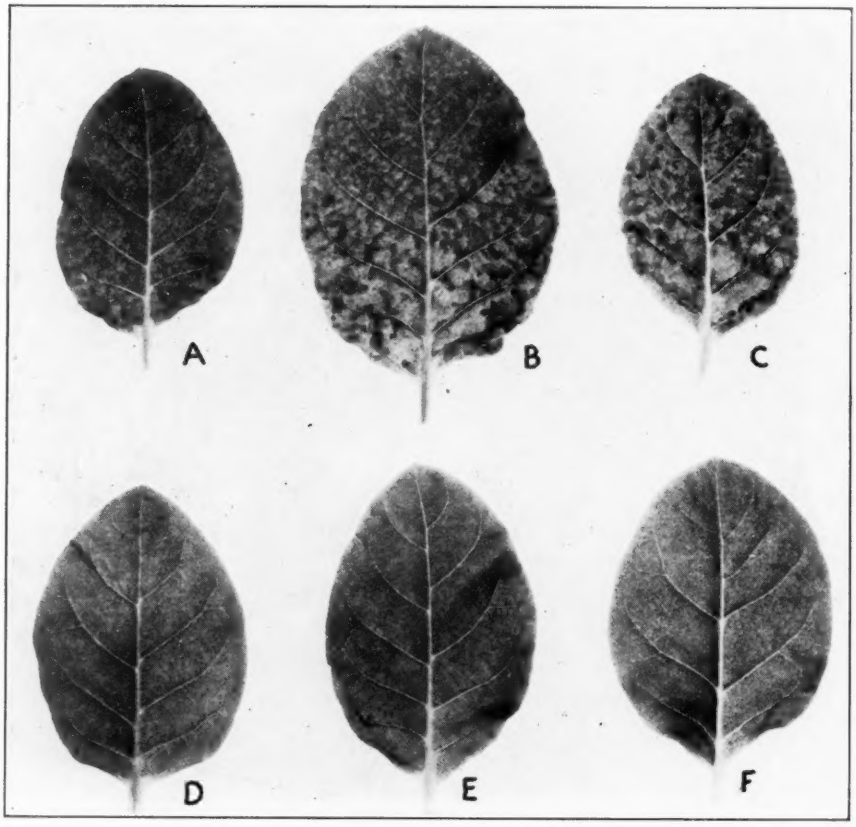

FIG. 5. A–C. Leaves of Turkish tobacco plants showing mixture patterns in plants infected with both distorting and mottling strains of tobacco-mosaic virus. D and E. Normal recovery in leaves previously affected by clearing of veins in plants infected with distorting (D) and mottling (E) strains of virus separately. F. Leaf of corresponding age from healthy plant.

Such spotted patterns never appeared in all plants receiving mixtures of strains. When two simultaneously introduced strains were about equally concentrated, approximately a third of the infected plants showed such mixture patterns. As the amount of either strain in a mixture was de-

creased, the proportion of mixture patterns likewise decreased. When one strain was 1/100 as concentrated as the other, about 5 per cent of the inoculated plants showed mixture patterns. All inoculated plants might reasonably be supposed to receive at least one infective dose of each strain in such mixtures. Failure to show a mixture pattern may be the result of occasional late systemic spread from primary lesions of one of the strains. It has been shown previously that the greater the initial infection with a single strain, the earlier, on the average, the systemic phase of the corresponding disease is manifested (1, p. 298), although a small initial infection may occasionally give an early systemic infection.

Observation of the spotted patterns on leaves that have previously shown clearing is a valuable aid in demonstrating contaminations of one strain with another. It may be used to supplement the methods of detecting mixtures of strains by transfers from single necrotic lesions and by transfers of minimum doses through pin-puncture inoculation.

Interference with Movement of Other Strains. The completely masked character of the masked-strain infection in tobacco and tomato suggests the possibility of using the masked strain of tobacco-mosaic virus to protect a plant against later introduction of the ordinary distorting strain of the same virus. Salaman (11) has described such a protective inoculation with the G form of his X virus against the more severe X form. Price (10) has described the modifying effect of ring-spot inoculation on subsequent infection with tobacco mosaic in tobacco. Kunkel (8) has recently described immunity from unattenuated aucuba and tobacco-mosaic viruses in *Nicotiana sylvestris* plants inoculated with attenuated strains derived from each.

Soon after isolation of the masked strain, its ability to protect plants from the effects of infection with distorting-strain tobacco-mosaic virus was tested. Several sets of tobacco plants were inoculated with the masked virus, an equal number of control sets remaining noninoculated. The distorting strain was then inoculated at once into the plants of one set already infected with the masked strain and into the plants of one control set. After intervals of one, two, and three weeks, it was similarly inoculated into other sets. In all cases, symptoms characteristic of infections with the distorting-strain virus finally appeared at the tops of the plants. Interference with the appearance of such symptoms was observable, however, upon comparison of the plants first infected with the masked strain with corresponding plants inoculated with the distorting strain only. In general, plants inoculated with the distorting strain after inoculation with the masked strain showed symptoms later than entirely unprotected plants receiving only the distorting strain. In some cases much more restricted, one-sided infections of the partly protected plants appeared at first, although upon further growth the symptoms characteristic of the distorting

strain always involved the whole top of the plant. The most pronounced action in blocking spread of the distorting-strain virus was found when the masked strain was introduced about one week before introduction of the distorting strain.

This effect of the masked strain in interfering with the movement of a distorting-type strain might, in spite of its incompleteness, be of considerable use in field practice. In the field, primary infections with virulent forms of the virus may be few and late. Inoculation of field plants with the masked virus, with its tendency to make large parts of the tissues of the plants unsuitable for the establishment of distorting-type infections, might reduce injury to the crop by the field type of virus. A preliminary test with tomatoes appeared to support this possibility. A planting of Bonny Best tomatoes in the garden was treated as follows: 23 plants were inoculated with masked-strain virus soon after setting out, 50 plants were inoculated with distorting-strain virus, and 22 were left noninoculated. The fruits were picked early, and weights of green fruit, ripe fruit, and stems with leaves were recorded. The results are shown in table 3. The plants inoculated with the masked strain of virus were distinctly better in average ripe-fruit weight, and slightly better in average yield of green fruit than

TABLE 3.—*Yields of Bonny Best tomato plants inoculated with masked and distorting strains of tobacco-mosaic virus, compared with yields of noninoculated plants*

	Plants exposed only to natural infection	Plants inoculated with	
		Masked strain	Distorting strain
Ripe fruits	0.16[a]	0.29	0.22
Green fruits	0.95	0.97	0.87
Stems and leaves	0.49	0.54	0.50

[a] Weight in kilograms per plant.

the set inoculated with distorting-strain virus or the set not inoculated at all, but exposed to chance infections in the garden during the growing season. It will be noticed that both inoculated groups of plants showed relatively early fruit ripening, *i.e.*, a large ratio of ripe to total weight of fruit at time of picking (Aug. 27), but that the total fruit weight was low in the plants inoculated with distorting-strain virus. Since no tomato varieties are known to be immune from injury by tobacco-mosaic virus, inoculation with a symptomless strain of this virus at planting time may be worth repeated trial.

Occurrence of Strains of Tobacco-Mosaic Virus in the Field. The masked, mottling, and distorting strains of tobacco-mosaic virus are similar

in many of their properties. A distorting type predominates in *Nicotiana tabacum* in the field. This predominance must indicate that the distorting type has exceptional ability to survive competition with other strains in this host.

The survival of the common distorting strain of virus in the field may depend in part upon its ability to spread to and multiply in young tissues. Starch patterns indicate that distorting-strain virus moves into young tissues and produces numerous secondary lesions in the systemic infection; attenuated strains seem to be more limited in their ability to pass into young leaves, apparently entering them later and at fewer points. Failure of the masked strain to protect completely against the distorting strain is perhaps a result of this incomplete occupation of young tissue.

If ability of virus strains to survive in the field is correlated with ability to move into young tissue of the host, one might expect certain plants to exert a filter-like action and to act as selective agents in isolating strains of peculiar properties. Something of this sort seems to occur in wild plants of *Nicotiana glauca*. The strain of tobacco mosaic common on *N. tabacum* tends to be symptomless and erratic in movement in *N. glauca*, as mentioned before. In wild plants of this species such a strain might not be successful because of the tendency of the plant to grow away from the infection. A strain of mosaic virus collected in California in a plant of *N. glauca* growing in an unfrequented spot was transferred to *N. glutinosa* and Turkish tobacco plants in the greenhouse. The necrotic primary lesions on *N. glutinosa* were indistinguishable from those of previously studied tobacco-mosaic strains, but the infections on Turkish tobacco were characterized by unusually pronounced stunting, late formation of new leaves marked by extreme distortion, and the formation of enations, or outgrowths of tissue from the lower sides of the leaves. These symptoms suggest ability to reach very young tissues of *N. tabacum*, because of the marked effect on the growth of the plant and on leaf development.

If this stunting strain enters and occupies very young tissue in *Nicotiana tabacum*, it may be inquired why it does not occur on this host commonly, to the exclusion of the typical field type. The excessive stunting of *N. tabacum* may seriously interfere, however, with chances for spread to other plants of the species. The reason for the dominance of the ordinary type of virus in *N. tabacum* may be that it combines relatively slight stunting of its host with considerable ability to displace slower-moving strains. The most favorable combination of such characters for any particular host would in time determine the common form to be found in that species under natural conditions. Maximum production of infected foliage to ensure frequent transfer from host to host, as well as extensive spread within the host plant to exclude other strains of the virus would be essen-

tial to continued existence. In *N. glauca*, the strain that unduly stunts *N. tabacum* is at no disadvantage, for it produces only mild symptoms and is able to move freely into newly-formed tissues to maintain infection in the individual plant.

ISOLATION OF ATTENUATED STRAINS FROM REPURIFIED STOCK OF DISTORTING-STRAIN TOBACCO-MOSAIC VIRUS

Tests of Virus Stocks for Purity

At the time of isolation of the original masked and mottling-type attenuated strains, it was believed that the distorting-strain stock was free of attenuated strains of the virus. Many characteristics of the attenuated strains and adequate techniques for demonstrating them in mixtures were not then known, however. In consequence, it was not possible to attempt to prove that these particular attenuated strains had been derived from virus of the distorting strain, from the stock of which they had been obtained. When study of known mixtures of strains had been completed, it was possible to isolate additional attenuated forms and to investigate the conditions allowing such forms to arise.

The stock of distorting-strain virus from which the first isolation of the masked strain was made had been obtained originally by diluting a supposedly pure, but untested, stock of tobacco-mosaic virus with a million parts of water to one part of juice of a mosaic plant. On 30 plants of *Nicotiana glutinosa* inoculated with this dilution, only 2 primary necrotic lesions appeared. A strain of virus was isolated by transfer from one of these two lesions to a plant of *N. tabacum*. From *N. tabacum*, transfer was made to plants of Bonny Best tomato; 35 days later the tomato plants were crushed and their juices diluted and frozen. When used to inoculate Turkish tobacco plants, this stock showed no indication (as would have been given by the appearance of mixture patterns, had such occurred in tests of the sample) of any admixture as great as 1:100 of an attenuated strain.

From this stock a new isolation was made by a similar process. A 1:1,000,000 dilution of the first single-lesion strain was used to inoculate 20 *Nicotiana glutinosa* plants. A lesion appeared on each of 2 of these plants. The leaf containing one of these lesions was cut into 11 portions, one containing the necrotic lesion, the other 10 consisting of green tissue only. Each of the 10 pieces of green tissue was crushed and the resulting extracts were each used to rub the surface of the leaves of a young Turkish tobacco plant. No disease developed from these inoculations. The 11th portion of the *N. glutinosa* leaf was crushed and transfer was made to 4 young Turkish tobacco plants. Three of these developed typical infections of distorting type. One of them was used as source of inoculum to infect 100 Turkish tobacco plants, the juices of which were preserved frozen.

From a consideration of the method of isolation, which experience has shown to separate two known strains when mixed together, even in equal quantities of expressed juice, it would appear improbable that any attenuated strains originally present in the old stock of virus would have been included in the repurified lot. Repeated tests of the stock immediately after its preservation confirmed this view by showing that mixture patterns were absent from 99 young Turkish tobacco plants inoculated with it. The same stock preserved by (1) freezing and (2) addition of 1 volume of glycerine to 1 volume of the diluted juice was tested again a little later by inoculation of 365 plants with a thawed sample of the frozen juice and by inoculation of 112 plants with the glycerine mixture, but no evidence of the presence of attenuated strains was obtained.

At the time of repurification of this stock of distorting-strain virus, a stock of tobacco-mosaic virus that had been kept frozen for about 8 years was found to give many mixture patterns. Upon minimum inoculation of a series of plants it was found to be a source of many attenuated strains, as well as of the supposed original distorting strain. This old stock did not furnish any completely masked strains, but about one-third of the isolations resembled the mottling type described in this paper, or were intermediate between this type and the distorting type. Naturally, nothing was known about the original purity of this stock, although it presumably represented typical tobacco-mosaic virus when first preserved. The source of the numerous attenuated strains obtained by direct minimal inoculation from it after it had been frozen about 8 years was, therefore, a matter of great interest, since the method of preservation might be considered to have induced their formation, and since the stocks that had been purified might also be considered in danger of spontaneous conversion to attenuated types after a time.

Aging of the frozen stock might have produced such attenuated strains. Tests of the first distorting-strain stock that had been carefully isolated were made. At the time of testing, this stock had been frozen 3¼ years. Two hundred young Turkish tobacco plants were inoculated by 5 pin punctures each, using this stock virus; 140 plants remained healthy, 60 showed distorting-type symptoms. Thus this stock of distorting-strain virus, after being frozen for more than 3 years, showed no evidence of admixture of attenuated types. No older, properly purified, frozen stock of virus was available. This same stock of virus, when it had been preserved frozen for 2 years, had been mixed with glycerine (10 volumes of glycerine to 1 volume of thawed undiluted stock of distorting-strain virus, and 9 volumes of water) and soon afterwards tested for purity of the distorting-strain virus by inoculating 321 Turkish tobacco plants. No evidence of admixture of attenuated strains in this stock had been obtained at that time. The

results of these experiments bring evidence that storage of virus in a frozen condition for two or three years does not induce the formation of detectable quantities of attenuated strains.

The original method of securing the impure stock might have been responsible for the occurrence of the attenuated strains. This stock had been obtained from old plants instead of from comparatively young ones, such as had been used for all of the more recently purified and tested stocks. The repurified stock was, therefore, introduced into young Turkish tobacco plants, and tests were made of the purity of the strain in these plants 4, 8, 13, 16, and 21 weeks after inoculation. On each occasion 100 to 200 healthy young plants were inoculated from various parts of the infected plants in the attempt to detect the presence of newly formed attenuated strains. At no stage was the presence of attenuated strains demonstrated, although all leaves, as well as stems, blossoms, and seed pods, had been included in the tests.

A little evaporation undoubtedly had occurred in the 8-year-old frozen stock of virus. Some residue, containing virus, must have been left on the sides of the tubes. This exposure of virus in dried condition might have induced the formation of attenuated strains. Dried leaf material of purified stock, about $3\frac{1}{2}$ years old, was ground in a little water, therefore, and tested for purity of the distorting-type virus contained therein. Turkish tobacco plants were inoculated by pin punctures, 5 to each plant. Among 360 inoculated plants, 103 distorting-type infections and 2 possibly attenuated infections were secured. A set of 128 similar plants, inoculated by rubbing, gave 121 typical distorting-type infections and 7 suspected mixture patterns. The 9 aberrant infections were transferred repeatedly to observe symptoms and isolate any attenuated strains. One of the two possibly attenuated strains proved to be the typical distorting strain, perhaps expressing its symptoms at first in an abnormal plant; the other appeared actually different from the distorting strain, but only slightly so, giving some distortion. From transfers of the 7 suspected mixture patterns, a single case of a definitely attenuated strain was finally isolated; this result suggests that drying for $3\frac{1}{2}$ years may have been effective in modifying the type.

The evidence for attenuation by drying seems hardly sufficient to account for the high proportion of attenuated strains in the 8-year-old stock; the origin of these strains, therefore, remains unexplained, since the more recently isolated frozen stocks derived from single lesions did not show evidence of detectable contamination of this sort.

Repeated Production of Attenuated Strains at High Temperature

Familiarity with the behavior of known mixtures of strains had made it possible to test the supposedly pure-strain frozen stocks, and it had been

found that these acted as though free from any appreciable contamination with attenuated strains. On the other hand, none of the tests could be expected to exclude the possibility that extremely small amounts of contamination might exist in such apparently pure stocks. It seemed necessary to repeat the work of isolation of attenuated strains in a quantitative way, to determine whether such strains could be isolated frequently after minimal inoculation.

In the following experiment an attempt was made to secure as many separate instances of attenuation as possible from a relatively few initial infections. About 120 pieces of stem, each about 1½ inches long, were cut from healthy Turkish tobacco plants. They were handled only with virus-free instruments and materials. A sample of each stem was tested by inoculation of *Nicotiana glutinosa;* no lesions appeared as a result of these inoculations, indicating that the stems were actually free from virus. The pieces of stem were inoculated with a 1:20 dilution of distorting-strain tobacco-mosaic virus, frozen since repurification. Five pin punctures were made in each stem, the pins being wet with inoculum before inserting them into the tissues. The inoculated stems were dropped into sterile test tubes, which were then stoppered lightly with cotton. These tubes were divided into two lots by putting alternate tubes into different wire baskets. All the tubes and their inoculated stems were held at a temperature of approximately 24° C. for 24 hours, before any were exposed to a higher temperature. This was done to aid in establishment of the minimal infections that had been given by the pin-puncture inoculation. Failure to allow some opportunity for establishment of virus at a temperature lower than 34° to 35° C. had been found to cause complete inhibition of all infections in certain earlier experiments. At the end of 24 hours, one lot of stems was allowed to remain at 24° C., the other was transferred to a water bath at 34.2° C. In order to improve the chances of establishment of all infections, the tubes exposed to high temperature were removed from the water bath at intervals during the first few days, being held at 24° C. with the control tubes for 6 hours on the third day, 7 hours on the fourth day, 5 hours on the fifth day, and 6 hours on the sixth day, as well as for the whole 24 hours of the first day after inoculation. Except for these periods, the high-temperature set of tubes was maintained at 34.2° C. in the water bath continuously until the eleventh day. It is not known that this program of alternating temperatures is essential, or the best one, but it did give the desired effect of establishing approximately the same proportion of infections in the stems treated at high temperature as in the control stems, as later test showed.

At the end of 11 days of incubation, the stems of the control set were crushed and their juices used to inoculate *Nicotiana glutinosa* plants. From

the resulting infections it was found that virus had multiplied at 24° C. in 55 of 60 inoculated stems. Thus, not less than 55 minimal infective doses of virus could have been concerned, since 55 separate stems became infected. If the assumption were made that the distribution of the primary infections among the 60 stems were by chance alone, a total of 168 minimal infective doses would have been the most probable number to have caused the 55 infections in 60 inoculated stems. Since any variability in stems or inoculation technique would have tended to increase this figure rather than to decrease it, it seems reasonable to assume a large number; it is judged that at the most several hundred minimal infective doses may have been introduced into this set of stems.

The stems of the set exposed to the higher temperature of 34.2° C. for a total of 9 of the 11 days following inoculation were also crushed individually and their juices used to inoculate *Nicotiana glutinosa* plants. From the resulting infections it was found that virus had multiplied in 49 of 55 inoculated stems of this set; this incidence of infection (89 per cent) was almost exactly the same as had been found among the controls (92 per cent). It may be assumed that all of the stems had originally received as great an average infection at time of inoculation as had the stems of the control set; therefore, it may be considered that several hundred minimal infective doses of virus were introduced into this set also.

Some of the primary necrotic lesions on the 49 *Nicotiana glutinosa* plants, infected from the set of stems incubated at 34.2° C., were used as sources of inoculum for transfers to young Turkish tobacco plants, 5 lesions from each original stem being used at first in most cases. Each lesion was ground separately and the resulting fluid was used to inoculate a single young plant of *N. tabacum* var. Turkish. Of the 49 sets of Turkish tobacco, each of 5 plants, all but 13 sets showed one or more infections with attenuated strains of tobacco-mosaic virus. An attempt was made to repeat the transfers for the 13 series that had yielded no attenuated strains, using additional necrotic lesions as sources of virus for test inoculation. From each of the 13 appropriate *N. glutinosa* plants additional necrotic lesions were isolated and crushed, and the juices were used as inoculum on additional young Turkish tobacco plants. Eleven days later it was found that 10 of these 13 new series showed one plant or more infected with an attenuated strain. By this time the original *N. glutinosa* leaves were not suitable for further use, and the three series that had yielded no attenuated strain were abandoned. Thus, of the total of 49 stems in which attenuated strains could have been produced, 46 were actually demonstrated to have produced an attenuated strain. Some of the series had produced more than one type of attenuated strain, many types from nearly masked to clearly mottling appearing in this experiment. The number of times that an at-

tenuated strain was produced from the several hundred original infections derived from the stock of repurified distorting-strain virus was in consequence more, rather than less, than 46.

Retesting of Stock; Absence of Attenuated Strains

The results of earlier study of the stock of distorting-strain virus had indicated that 46 or more isolations of an attenuated strain could not be expected in a few hundred minimal infective doses demonstrated by direct inoculation from it. In view, however, of the ever-present possibility that the passage of time might affect the purity of the frozen stocks, it seemed desirable to retest the particular stock used in this experiment and compare it with known mixtures of strains after completion of the attenuation experiment.

The thawed sample of virus stock prepared for the original stem inoculation in this experiment had been divided into two portions, and one of these had been refrozen as a control on the continued purity of the stock virus, the other having been used in the inoculation of stems. When the results of the experiment were known, the refrozen portion was thawed and tested to determine whether it was still free from attenuated strains. Successive tests were conducted as follows.

Young Turkish tobacco plants were inoculated with the residue of the 1:20 stock on three occasions, the inoculum being thawed and refrozen each time. Each plant was punctured with a set of 5 insect pins inserted near the base of a young leaf. In the first set 100 plants were inoculated, 42 showed symptoms typical of infection with the distorting strain, none showed mild-mottling symptoms. In the second set 150 plants were inoculated, 63 showed distorting-type symptoms, none showed mild symptoms. In the third set 150 plants were inoculated, 81 showed distorting-type symptoms, but none showed mild symptoms. Thus, in 400 inoculated plants, 186 typical infections of the distorting type arose, but no attenuated strain was found.

As a control, a mixture was made of 10 parts of the 1:20 stock distorting-strain virus with 1 part of the 1:20 mottling-strain stock. In a set of 100 Turkish tobacco plants inoculated with this mixture, using 5 pin punctures in each plant as before, 51 plants remained healthy, 46 plants showed distorting-type symptoms, 2 plants showed mottling of the mild type without leaf distorting, and 1 showed a mixture pattern, indicating infection with both strains of virus. The success in isolation of an attenuated strain from a 1:10 mixture by this method of minimum inoculation indicated that no contamination of the original stock could have approached this 1:10 ratio. It will be realized that this method of minimum inoculation could not be used to demonstrate the presence of an attenuated strain of virus in

smaller ratios to the distorting-strain virus, as in the ratios 1 : 100 to 1 : 1000, without the use of very large numbers of test plants. A more sensitive test of the presence of admixed attenuated strains was at hand, however, in the form of the test for the production of mixture patterns.

The mixture-pattern test was applied to a portion of the original stock refrozen after the attenuation experiment was started. One hundred Turkish tobacco plants were inoculated with the supposedly pure distorting-strain stock. These plants were rubbed with a glass spatula to give a heavy inoculation from the 1 : 20 dilution of juice. Another 100 plants were inoculated similarly, but with 1 part of 1 : 20 mottling-strain stock purposely mixed with 1000 parts of the 1 : 20 distorting-strain stock (proportion of strains, 1 : 1000). A third 100 plants were inoculated with a 1 : 100 mixture; a fourth set involved a 1 : 10 mixture. A fifth set of a hundred plants was reserved for inoculation with the mottling-strain stock alone. As a result of the inoculation, all plants except one of the mottling-strain set became infected. It is believed that this plant may have been left uninoculated. At the end of two weeks, the first set showed no mixture patterns, but 100 typical distorting-type infections; the second showed 19 mixture patterns; the third and fourth sets showed 5 and 1 mixture patterns, respectively; the fifth set showed no mixture patterns, nor distorting-type infections, but all mottling-type infections, except for the one plant that appeared to have escaped inoculation. Evidently the residue of the original stock of distorting-type virus used in the attenuation experiment had not contained so much attenuated virus as did a purposely made mixture of 1 part of mottling-strain stock in 100 parts of distorting-strain stock.

DISCUSSION

With present methods it is not feasible to demonstrate that very small traces of attenuated strains are not present in such a stock as the supposedly pure distorting-strain stock described in this paper. Evidence has been given, however, for believing that the distorting-strain stock did not contain so much of attenuated strains as would a mixture of a hundred parts of this stock and one part of a mottling-type stock of similar virus concentration. The isolation of at least 46 attenuated stocks from a few hundred minimal infective doses derived from the original distorting-strain stock of tobacco-mosaic virus furnishes some evidence, therefore, for the contention that the appearance of attenuated strains of tobacco-mosaic virus during incubation of the ordinary distorting strain in plant tissue at a temperature of a little above 34° C. is not to be ascribed to selection of some contaminant in the original virus stock but is a result of the action of high temperatures on the distorting-strain virus. It is not contended that failure to find such derivatives in purified stocks of the distorting strain as a result

of exposing them to freezing or long standing in tissues of living plant hosts indicates that attenuated strains do not sometimes appear under these conditions. The experiments herein reported have merely shown that the distorting strain of tobacco-mosaic virus is relatively stable under these conditions, yet readily produces attenuated strains during incubation in living tissues at temperatures just above 34° C., as originally indicated by Johnson (5) in 1926.

The identity of host range, so far as tested, and constant relationship of symptoms of the distorting, mottling, and masked stocks of tobacco-mosaic virus are striking. This confirms the evidence for close relationship of these strains to each other, as given by the experiments concerned with the derivation of one type from another. Other viruses, such as those of cucumber mosaic, mottle of potato, bean mosaic, raspberry mosaic, etc., are not known to show corresponding similarities over an extended host range and are believed not to be closely related to each other or to tobacco-mosaic virus. It seems appropriate to refer to such stocks as have no observable similarity in symptoms over an extended host range as separate viruses, but to those in which an evident relationship of symptoms occurs over an extended host range as strains of a single virus. This practice has been followed in the present paper. The intended distinction is comparable to that between species and varieties among plants.

The clearly recognizable characteristics of the three strains of virus described in this paper may lend assistance in the study of the fundamental question of virus nature. Accumulated evidence concerned with the behavior of tobacco-mosaic virus is interpreted by some as indicating that the virus is of the nature of an organism, by others as indicating that the virus is a single chemical compound. It is, of course, also possible that the virus may represent a primitive, or a degenerate, obligate parasite, consisting of an organized group of a relatively small number of chemical compounds. Whatever the nature of the virus, whether a complex living organism, a single chemical compound, or a small organized group of chemical compounds, the study of the conditions allowing the transmutation of one strain to another may prove to be important. It has been shown that many properties of the three strains of tobacco-mosaic virus described in this paper are alike for all strains, and that there are a few distinctive properties. If the virus were a chemical compound, the strains would necessarily represent related new compounds or isomers. If a chemical treatment could be found that would consistently change one of these strains into another, it would probably be feasible to develop the quantitative study of the reaction because of the opportunity to estimate the concentration of each strain. If the virus were a primitive or a degenerate organism, the possibility that a simple relationship, even a simple chemical relationship, might exist between the

strains is not entirely excluded. It is, indeed, among the viruses, if anywhere, that one might look for living matter so simple in constitution that its constituent materials might be few enough to be readily studied.

The difficulty of invasion of very young tissues by the masked strain of virus raises an important theoretical consideration. The difference in fundamental nature of the masked strain and distorting strain may be of such a sort as to make it easier for the plant to restrain movement of masked-strain virus within leaf tissues. There is a possibility that study of this characteristic of the masked strain may later add to our knowledge of virus nature some information on the particular way in which these strains differ from each other.

SUMMARY

A completely masked strain of tobacco-mosaic virus, possessing many properties of the ordinary distorting, field-type virus, was studied in comparison with a typical mild-mottling strain and with the distorting strain from which it appeared to have been derived. Mixtures of the distorting strain and the masked and mottling-type attenuated strains could be distinguished with ease from single-lesion, presumably pure, stocks of the distorting strain when as little as one per cent of the admixed type was present. It was shown that stocks of distorting-strain virus, acting as though not containing admixtures to this extent and presumed to be entirely free from attenuated strains, remained free from such detectable admixtures of mild-mottling types in plants and in frozen juice for considerable periods of time. Small inoculations of such stocks of distorting-strain virus into healthy stem tissues incubated at temperatures a little above 34° C. gave rise frequently to attenuated strains. The evidence points to the appearance of new and stable strains from the old stock.

The differences found between the original and the derived strains were few in comparison with their common properties. The masked, mottling, and distorting strains were similar in host range, approximate thermal death point, ability to remain infective after long storage, infectivity in undiluted and diluted juices, production of necrotic primary lesions identical in size, form, and general appearance in certain hosts, and starch-retention patterns in inoculated leaves of certain other hosts. The strains differed principally in two respects. The attenuated strains were able to increase in host tissues at temperatures high enough to inhibit multiplication of virus of the original distorting strain. They caused less chlorosis in the mottling patterns and appeared to involve tissues near the growing point later than did the typical distorting-type tobacco-mosaic virus. In the latter respect the relation between the masked strain and *Nicotiana tabacum* resembles the relation previously shown to exist between the distorting strain

of tobacco-mosaic virus and its symptomless carriers, which do not show yellowish primary lesions at the site of infection and which do not allow this strain to enter, or else do not allow it to come to high concentration in, cells of young tissues.

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THE CYTOLOGY OF UROCYSTIS OCCULTA[1]

E. C. Stakman, R. C. Cassell, and M. B. Moore

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INTRODUCTION

As part of a comprehensive investigation of *Urocystis occulta* (Wallr.) Rabh., the writers studied the cytological phenomena as a basis for a study of the possible origin of races through hybridization.

The cytology of several species of Urocystis has been investigated by several investigators, notably Paravicini, Kniep, Rawitscher, and Blizzard.

Paravicini (4) studied *Urocystis anemones* (Persoon) Winter and *U. violae* (Sow.) Fischer v. Waldh. According to him, long, several-celled mycelial threads are abjointed from the tips of promycelia of *U. anemones*. The cells of these threads are uninucleate and the dikaryophase arises from the fusion of neighboring cells in the same mycelial thread. Paravicini states that the fusions were difficult to see and that there probably are other types of fusions, also. According to his observations, the sporidia of *U. violae* are readily abjointed from the promycelium and then fuse in pairs. Kniep and Rawitscher, however, do not agree with Paravicini.

Kniep (2, 3) investigated *Urocystis anemones*. He illustrates a short promycelium with several uninucleate sporidia, which do not become abjointed. The sporidia fuse in pairs at the base, the nucleus from one passes into the other, which then elongates into a binucleate hypha, thus initiating the dikaryophase.

Rawitscher's observations on the origin of the dikaryophase in *Urocystis violae* agree with those of Kniep on *U. anemones*. Rawitscher (5) states that the origin of the binucleate condition was hard to determine, because 8 sporidia usually are formed and are so crowded at the base that the fine fusion tubes are difficult to see. He states that the binucleate sporidia may become abjointed from the promycelium and bud, the 2 nuclei undergoing conjugate division. In this respect, then, *U. violae* apparently is different from *U. anemones*.

Blizzard (1) found no evidence of fusion between promycelial branches of *Urocystis cepulae* Frost. These branches may become multicellular, but each cell contains a single nucleus. Blizzard states that the saprophytic mycelium "is uninucleate from the beginning and remains so throughout

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its existence." He states that the cells of the parasitic mycelium also are uninucleate at first but "become progressively binucleate as they approach the young sorus primordium, where all the cells of the sporogenous hyphae are binucleate."

On the basis of these reports there appears to be considerable variation and variability between and within these 3 species. The observations of Kniep and Rawitscher agree in essentials. The apparently conflicting results of Paravicini and Kniep on *Urocystis anemones* may be due to differences in conditions under which spores were germinated, although both authors agree that fusions occur and are sexual in nature. Blizzard's observations in *U. cepulae* appear to be an exception to the widely accepted view that the fusion of sporidia or promycelial cells initiates the dikaryophase. It is well known, however, that the dikaryophase of most smuts does not maintain itself well on artificial media; hence, one would expect that *U. cepulae* might be in the haplophase when grown on onion agar, as Blizzard found it to be. The lack of fusions between promycelial branches might be accounted for in the same way, although there is no reason why all species of Urocystis should necessarily behave alike in this respect. A comprehensive study of different species under various comparable conditions is desirable.

SPORE GERMINATION

Wolff (6), as early as 1873, described and illustrated spore germination in *Urocystis occulta*. He states that the spores require from 3 to 4 days to germinate, that they send out a promycelium through a split in the wall, and that 2 to 6 sporidia are formed. He noted, further, that very rarely certain sporidia were united by "eine Art von Brücke"—a kind of bridge; and he figures a distinct H-shape fusion, but does not show the nuclear condition.

In general, the writers' observations agree with those of Wolff. In our experiments the spores germinated best after having been soaked for about 16 hours in water to which benzaldehyde had been added at the rate of 3:2,000,000. Germination tests were made at various temperatures, ranging from 9.5° to 28° C. Early germination was best at about 15°, but 24° was more favorable for subsequent development. Details of the relation of temperature to spore germination and the development of smut will be discussed in a separate paper.

Under favorable conditions spores usually germinate well in from 30 to 48 hours. The spore wall is split and a promycelium is sent out. It may be short and nonseptate or may grow to considerable length without forming cross walls. Sometimes, however, it becomes septate and may consist of as many as 10 to 15 cells, the protoplasm usually being aggregated in the tip cell.

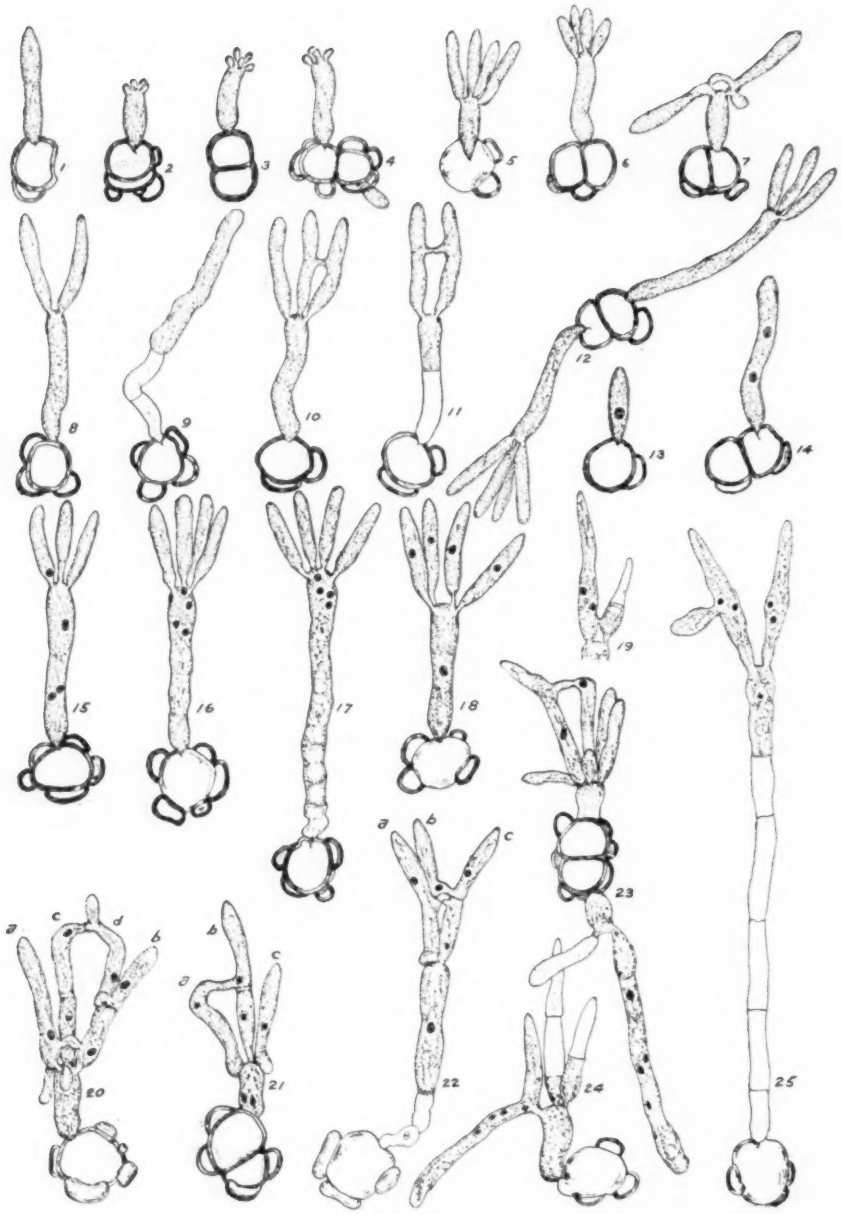

PLATE I

Sporidia begin to form within a few hours after germination. They are somewhat variable in size and shape, and quite variable in number and in time of formation. Often they begin to form when the promycelium has attained a length of only 20 or 30 μ, but they may not begin to develop until it is many times as long (Plates I and II). The number ranges from 2 to 6, 4 being a very common number. Sometimes there appear to be still more, but in such cases some are small and possibly should not be considered as sporidia. Relatively short, thick protuberances often grow out from the base of the whorl of sporidia, either from the promycelium itself or from the base of the sporidia.

The sporidia, as far as we have been able to observe, remain attached to the promycelium and do not function as conidia, although further study

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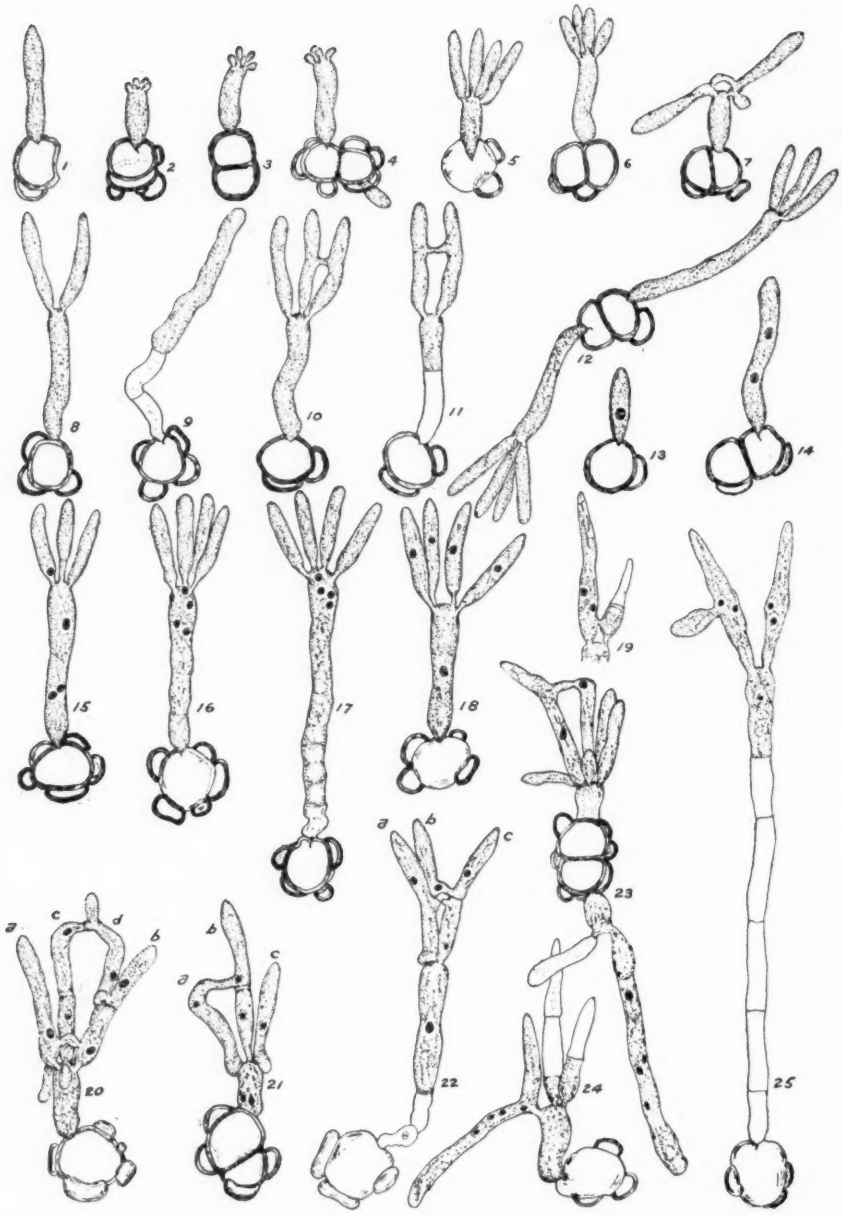
PLATE I

Stages of spore germination of *Urocystis occulta* in water. 1–6. Young promycelia showing the formation of sporidia. 7. Basal fusion between two sporidia. 8–9. Later stages. 10–11. H-shape fusions between sporidia. These are fairly common. 12. Germination of a 2-celled spore, each fertile cell of which has sent out a rather long promycelium.

Development on oat-meal agar. 13–14. Young promycelia, showing a single large nucleus in 13 and two nuclei in 14. 15–17. Promycelia on which the sporidia are completely or almost completely formed; nuclei about to pass into the sporidia. 18. Each sporidium has received a single nucleus, and there is one in the promycelium. 19. Fusion between two sporidia on the promycelium. The nucleus from the one on the right has passed to the sporidium on the left, which has begun to elongate, while the one on the right is becoming vacuolate. 20. Fusions between sporidia. Sporidia *a* and *b* have fused near the base; *b* has become septate, each cell containing a nucleus; the upper cell is sending out a protuberance that probably will fuse with the lower cell in *c*. The tips of sporidia *c* and *d* have fused, and a hypha is being sent out from *d*, into which the nucleus from *c* is moving. Sporidium *c* has become 2-celled, each cell with a single nucleus. The basal cell appears to be sending out a protuberance near the septum, preparatory to fusion with the upper cell of *b*. Short, bulbous branches, such as those shown at the base of the sporidial whorl, are fairly common. 21. Sporidia *a* and *b* have fused and *b* is beginning to elongate. A septum has been laid down just below the fusion tube in *b*. The lower cell contains a nucleus and appears to be about to fuse at the base with *c*. There appear to be two nuclei in the promycelium. 22. Sporidia *b* and *c* have fused. The nucleus from *b* is passing into *c*, which has formed a cross wall just below the fusion tube, and the lower cell is about to fuse with sporidium *a*. 23. Two sporidia on the upper promycelium have fused and the one on the left is beginning to send out a hypha. Evidently, fusion has already occurred on the lower promycelium, but it could not be seen. 24. Four nuclei from the promycelium apparently have passed directly into the hypha growing out from the sporidium on the left. There is no evidence of fusion, and the two sporidia on the right are degenerating. Apparently the fungus has taken a short cut in initiating the dikaryophase, and the nuclei have become paired without previous fusion between sporidia. 25. A long, septate promycelium, the protoplasm aggregated into the tip cell on which each of the two large sporidia contain two nuclei, presumably derived from the promycelium directly, as there is no evidence of fusion.



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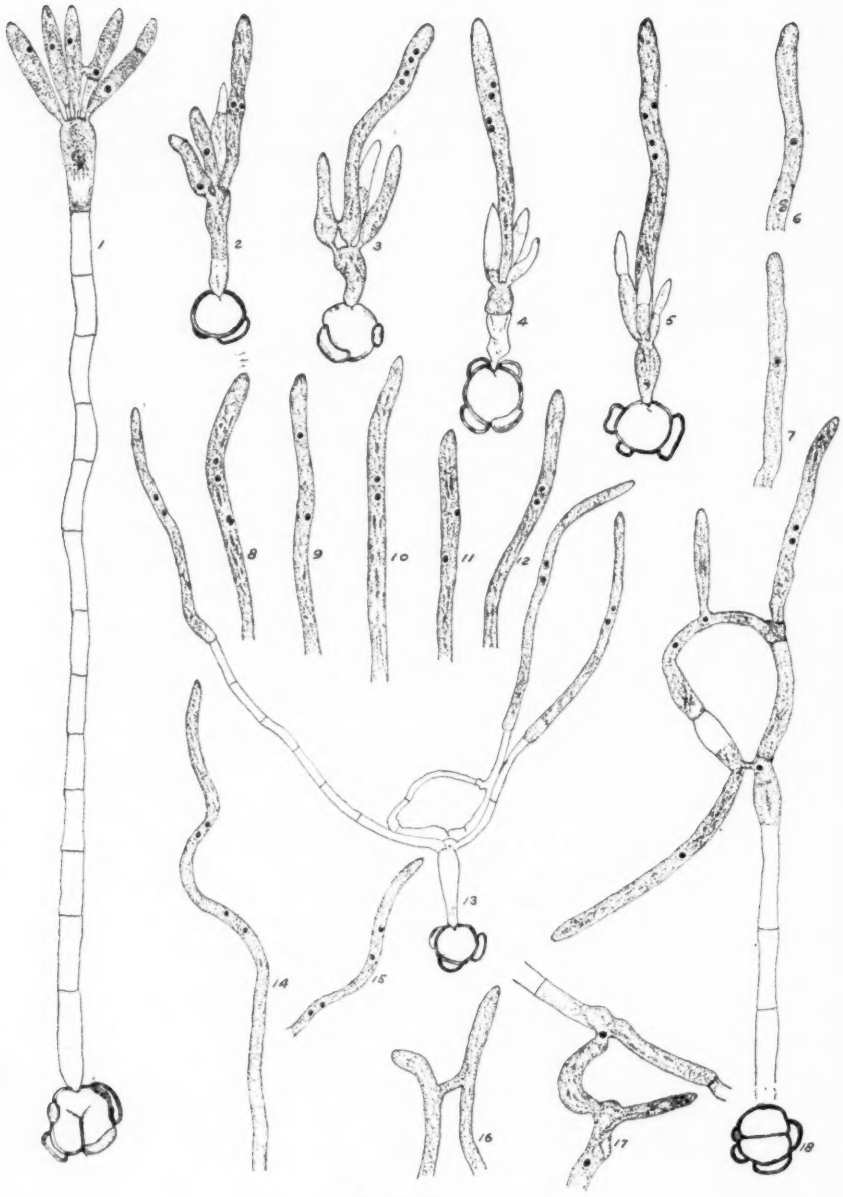

PLATE II

of their behavior under different conditions is desirable. Apparently, however, they represent a greatly shortened haplophase in the life cycle of the organism. They grow, receive a haploid nucleus from the promycelium, fuse with another sporidium, and either degenerate or send out a dikaryotic hypha, depending on whether they are on the delivering or receiving end of the fusion tube. If they do not fuse, they seem to degenerate in most cases. The haplophase, therefore, seems to be limited in function as well as in duration, under the conditions of the writers' experiments.

NUCLEAR BEHAVIOR

Germinating Spores. Spores were dusted on a thin film of oatmeal agar on slides, which were then placed in a moist chamber until the desired stage of development of the fungus had been reached. The material was then killed in Flemming's weaker solution, washed and fixed, and stained with iron-alum haematoxylin.

The normal nuclear behavior may be summarized as follows. The young promycelium contains a single, large nucleus, in which a nucleolus usually is clearly visible. Successive nuclear divisions occur, resulting in the for-

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PLATE II

The development of the dikaryophase. 1. A somewhat unusual type of promycelium, comprising many empty cells and one cell at the tip into which the protoplasm has been aggregated. Two of the sporidia have fused. 2–5. Early stages in the development of dikaryotic hyphae, such as result from the fusion of sporidia. In 2 the sporidium, which is becoming vacuolate, evidently has fused with the one that is elongating into a hypha, although the fusion tube was not visible. In 3 a fusion tube is clearly visible. In 4 and 5 a single hypha has been sent out in each case, and the other sporidia are degenerating. The nuclei are arranged in two pairs. 6–12. Hyphal tips, such as those shown in 13. In 6 there are three nuclei. Apparently the lower pair have just resulted from division, while the upper nucleus has not divided. It is assumed that the two original nuclei did not divide at exactly the same time. 7, a hyphal tip in which a single nucleus only was visible. In 8 the upper pair of nuclei probably have resulted from the division of a single nucleus, while the lower pair are just separating. A short time earlier this hyphal tip probably would have appeared to be 3-nucleate. 13. A promycelium on which three dikaryotic hyphae have developed; fusion tube between two of the sporidia still visible. 14–15. Tips of very long hyphae such as those shown in 13, with the nuclei distinctly arranged in two pairs. 16. H-shape fusion between hyphal tips. Unfortunately, the nuclei were not visible. 17. Fusion between the tip cells of two long hyphae that appeared to be uninucleate. The nucleus from the upper one is passing into the lower one which already is sending out a hypha. These hyphae could be traced through a large number of empty cells to different clumps of spores. 18. The spore has sent out a long germ tube, some of the empty cells of which are not shown. Two of the cells have become binucleate and are sending out hyphae, while the tip cell has turned backward and fused with the first living uninucleate cell. The nucleus from the latter appears about to pass through the fusion tube into the tip cell, which apparently has begun to elongate into a hypha.

mation of 4 or more nuclei, 4 and 6 being the most common. Normally, one nucleus passes into each sporidium, often after the sporidia have attained almost their maximum size. Regardless of the number of sporidia, each contains a single nucleus prior to sporidial fusions, with the exceptions noted below. A nucleus may or may not be visible in the promycelium after each sporidium has received one (Plate I, 13 to 18).

As soon as the sporidia are fully formed they tend to fuse (Plate I, 19 to 23, and text Fig. 1). There seems to be no fixed method for fusion. H-shape fusions, such as those in *Tilletia* spp., occur frequently (Plate I, 10, 11, 19, and 22; Plate II, 1 and 3); basal fusions, as described by Kniep for *Urocystis anemones*, are common (Plate I, 7 and 20, and text Fig. 1, A and B); and tip fusions occur frequently (Plate I, 20, 21, and 23, and text Fig. 1, C and D).

Not only is the method of fusion variable, but the nuclear behavior, also, varies somewhat. The simplest case is shown in plate I, 19. There were only 2 sporidia on the promycelium; they have become united by a fusion tube; and the nucleus from the sporidium on the right has passed into that on the left, which has begun to elongate into a hypha. The sporidium on the right already is partially vacuolate. In plate I, 23, two sporidia on the promycelium from the upper cell of the spore have fused at their tips; and the nucleus from the one on the right is passing over to the one on the left, which already has begun to send out a hypha. The sequence of events is slightly more complex in 20, 21, and 22 of plate I. In 22 there is a fusion tube between sporidia *b* and *c;* the nucleus from *b* is just passing through the fusion tube into *c,* in which a septum has been formed just below the fusion tube. The lower cell of *c,* which appears to contain a nucleus, is about to fuse with the third sporidium, *a*. It is not entirely clear whether the nucleus of sporidium *c* divided, as one could not be certain that the dark-staining body near the base is a nucleus. If it is not, presumably the nucleus in the promycelium would move up. In 21 there is a similar situation. Sporidia *a* and *b* have fused near their tips, and *b* already is elongating into a hypha. A septum has been formed just below the point of fusion in *b,* and the lower cell probably will fuse with sporidium *c,* which appears to be sending out a protuberance at the base. Since young, nonfused sporidia are almost universally uninucleate, it is probable that sporidium *b* originally contained a single nucleus that subsequently divided, giving rise to 2 sister nuclei, although it is possible, of course, that 2 nuclei migrated directly from the promycelium into the sporidium. In 20, sporidia *a* and *b* have fused near the base by means of a broad fusion tube, while the tips of *c* and *d* have fused and a hypha is growing out from *d*. Cross walls have formed in *c* and *b* and the upper cell of the latter and the basal cell of the former appear to be about to fuse, as there is a slight protuber-

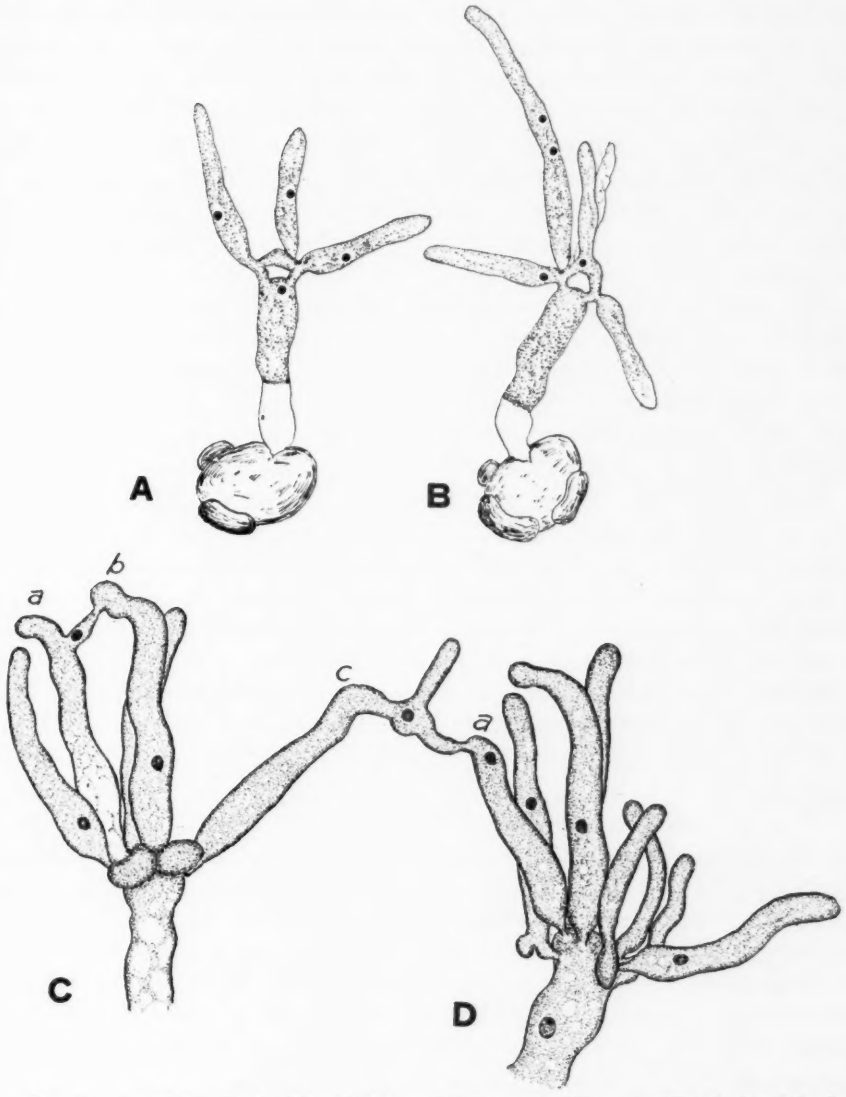

FIG. 1. A and B. Typical basal fusions between sporidia. A. The fusion tube between two sporidia is complete, but the nuclei have not yet paired. B. Two sporidia connected by a fusion tube that is sending out a hypha into which the two nuclei apparently are passing. The other two sporidia evidently have fused also; one is binucleate and is elongating into a hypha. The connection could not be seen, however. C and D. Two promycelia showing sporidial fusions. On promycelium C sporidia *a* and *b* have fused; the nucleus from *a* already in the fusion tube and passing over toward *b*. There are basal fusions between some of the sporidia also, but the exact connection could not be made out. Sporidium *c* of promycelium C has fused with sporidium *a* of promycelium D by means of a long fusion tube, which is already sending out a hypha. The nucleus from *c* is about to enter this hypha, while that from *a* is about to pass into the fusion tube. There is a large nucleus in promycelium D; note nucleolus.

ance on the *c* and a pronounced one on the *b*. For the sake of clearness, these 2 sporidia are shown farther apart than they originally were. During the extremely hot weather of August the balsam on the slide became soft, and by pressing the cover slip gently the sporidia could be made to move into almost any desired position. They were drawn in the most favorable position for showing fusions; hence the likelihood of fusion between *c* and *b* does not seem so strong from a study of the drawing as it did from a study of the material itself. Sporidia *a* and *c* would seem to be of one sex and *b* and *d* of another.

Sporidia from different promycelia may fuse also, as shown in text figure 1. A hypha already has begun to grow from the swollen portion of the rather long fusion tube between sporidium *c* of promycelium C and sporidium *a* of promycelium D. Sporidia *a* and *b* of promycelium C also have fused, and it is likely that there are basal fusions between some of the other sporidia, also, but it was not possible to distinguish them clearly.

Very soon after fusion, even before the nuclei have become closely paired, one of the fused sporidia, or the fusion tube itself, sends out a hypha, while the other sporidium becomes vacuolate and often collapses, as is true also of the nonfused sporidia. Early stages in the development of the hyphae are shown in the figures in plate I, already alluded to, while somewhat later stages are shown in plate II, 2, 3, 4, 5, and 13.

From 1 to 3 hyphae develop on a single promycelium. Usually they are binucleate in the early stages, but later there often are 2 pairs of nuclei. In a few hyphae there appear to be 3 nuclei, and very rarely there seems to be only one.

Nuclear counts were made in 100 hyphae on several slides on which various stages of germination were represented. Similar hyphae are shown in plate II, 6 to 15. In one hypha there was one nucleus; in 47 there were 2; in 4 there were 3; and in 48 there were 4.

Ninety-five per cent of the hyphae, therefore, had either 2 or 4 nuclei, often arranged distinctly in pairs, as shown in plate II, 14 and 15. Those having 3 nuclei may have been in the dikaryophase, also, but were temporarily 3-nucleate because the 2 original nuclei did not divide at exactly the same time. In plate II, 6, for example, the lower nucleus of the pair has just divided, while the upper one has not yet divided; and in plate II, 8, the upper nucleus apparently has divided and the daughter nuclei are a short distance apart, while the lower nucleus is just completing division, the daughter nuclei still lying close together. A few minutes earlier this hypha probably would have had 3 nuclei. It is possible, too, that the fourth nucleus sometimes is not visible, as it is only in the best-stained slides that all nuclei can be seen clearly. Because of these facts and the fact that the young hyphae are almost universally binucleate or 4-nucleate (Plate II,

2 to 5), it seems most probable that the older hyphae in which 3 nuclei were counted either were only temporarily in this condition, owing to a slight difference in the time of division of the 2 original nuclei, or that the fourth nucleus was not visible, either because of imperfect staining or possibly because of the position of 2 nuclei in approximately the same vertical plane, one superimposed on the other. If this is true, the percentage of hyphae in the dikaryophase was 99 per cent and only 1 per cent appeared to have a single nucleus. Here again, however, the second nucleus may not have been visible for the reasons mentioned above or the hypha may have been binucleate at first and may later have become uninucleate.

That a few hyphae actually are uninucleate is suggested by such conditions as shown in plate II, 7 and 17. The hyphal tip shown in 7 was well stained and the only visible nucleus was very distinct. The 2 hyphae shown in 17 were uninucleate. They could be traced back, along many empty cells, to different clumps of spores, and the 2 cells at the tips have fused; the nucleus from the upper hyphae is passing into the lower one, which is sending a hypha out toward the right. It seems probable, therefore, that older hyphae may occasionally be uninucleate and may fuse with each other to produce binucleate hyphae.

Apparently the dikaryophase sometimes is initiated without previous fusion. (See Plate I, 24 and 25.) In both of these cases there was no evidence whatever of fusions; nevertheless, in 24 the sporidium on the left has sent out a hypha that contains 4 nuclei, which apparently came directly from the promycelium. The other 2 sporidia contain no visible nuclei and are degenerating. In 25 the 2 large sporidia are binucleate and there is no indication whatever of fusions, as was agreed by at least 6 competent observers. In plate II, 18, is shown a promycelium that grew directly into a long germ tube, 2 of the cells of which are binucleate and are sending out hyphae, while the uninucleate end cell has curved backward and is fusing with another uninucleate cell.

It is clear from the above that the dikaryophase may originate in several different ways. The normal condition is for the uninucleate sporidia of the same promycelium to fuse, either at the base, or by means of H-shape fusions, or at the tips; but sporidia of different promycelia also may fuse. Occasionally, rather long, uninucleate hyphae apparently develop, and these may then fuse and send out a typical dikaryotic hypha. In one case, at least, all the nuclei from the promycelium apparently passed directly into one sporidium, which then developed further, while the others disintegrated. In still another case 2 sporidia on a promycelium each contained 2 nuclei, and, as there were only 2 sporidia on this promycelium and they had not fused, it is presumed that the nuclei paired before passing into the sporidia, although it is possible that each sporidium originally contained one

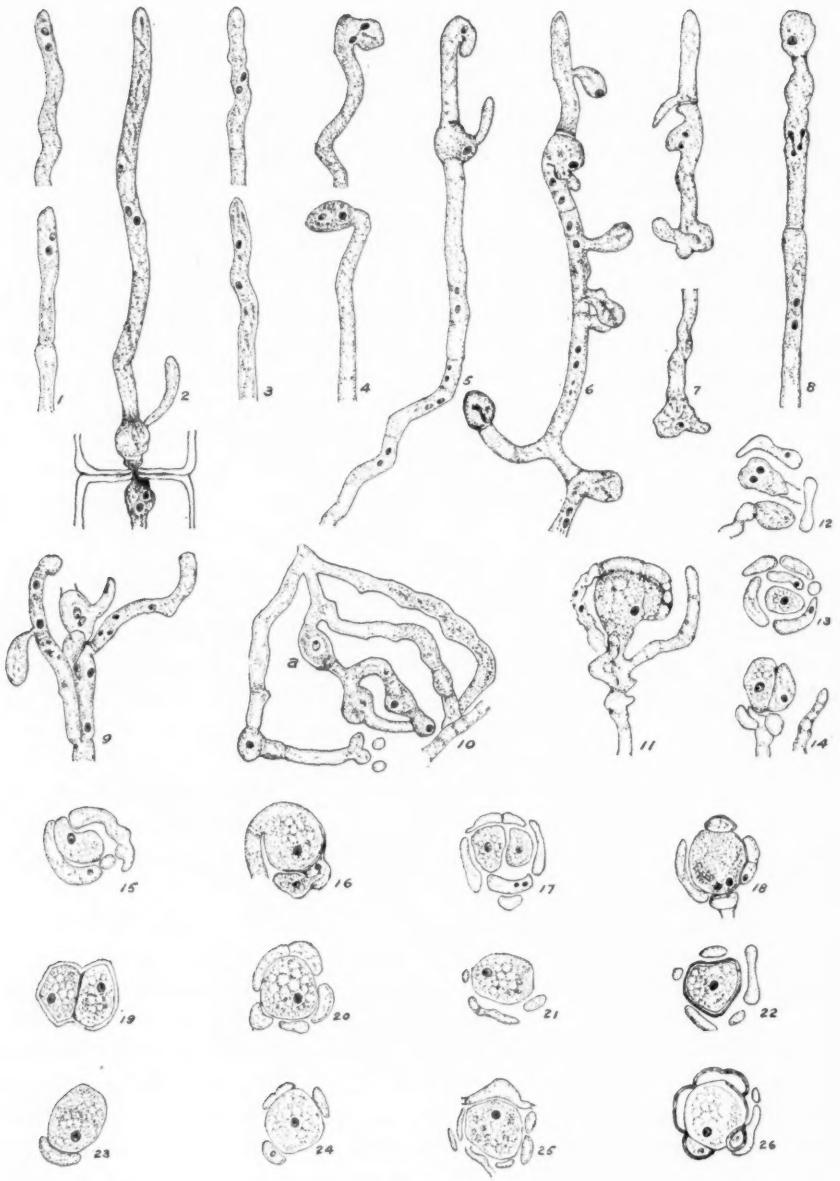

PLATE III

nucleus, which divided in the sporidium. One promycelium was observed that grew out directly into a long germ tube in which 3 cells became binucleate, one by fusion with another cell of the same tube.

Apparently, therefore, several haploid nuclei ordinarily result from the division of a single diploid nucleus in the young promycelium. These haploid nuclei normally pair after fusion of sporidia, and exceptionally, perhaps, after fusion between uninucleate hyphae. It seems significant that the characteristic dikaryotic hyphae begin to develop very soon after plasmogamy. The presence of 2 nuclei in a protoplast, even when they are still a considerable distance apart, imparts to it an ability that the haplophase does not seem to possess.

It would seem that infection of the host must result from invasion by hyphae of the dikaryophase, as only such hyphae seem to be capable of appreciable development. As far as our observations go, 48 hours after the spores began to germinate there were virtually no hyphae except those of the dikaryophase. The writers are quite aware, however, that there is considerable variability in the development of smut fungi and would not

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PLATE III

The mycelium in the host and spore formation. 1–3. Vegetative hyphae in the rye plant, showing the binucleate condition. In 2 the mycelium is intracellular and has forced its way through the transverse wall between two parenchyma cells. Both the lower cell and the upper cell are clearly binucleate. 4. Swollen tips of hyphae that are still binucleate. 5. A hypha in which the basal cells are binucleate, and two large uninucleate cells apparently rounding up into spore mother cells. 6. A bit of mycelium in which there are four successive binucleate cells and two uninucleate ones, the lower of which evidently is a spore mother cell. 7. Above, a cell beginning to swell up into what may become a spore mother cell; below, a tip cell that has become uninucleate. 8. The basal cell is distinctly binucleate. The cell immediately above it contains two nuclei that seem to be in process of division, while the upper cell is distinctly uninucleate and is probably a spore mother cell. The hypha may have continued beyond the upper cell, but, because of its gnarled condition, could not be traced farther. 9. A spore mother cell in which the nuclei are about to fuse; hyphae, which presumably will form the sterile cells, growing up around it. 10. Sporulating mycelium. Cell *a* appears definitely to be a spore mother cell. It is probable that some of the surrounding hyphae may become attached as sterile cells. 11. A later stage in spore formation. Apparently, the next to the last cell in the hypha is rounding up into a spore mother cell, while the tip cell has curved downward and may also become a fertile cell. The hyphae from below are curving around these cells, some of them already having become appressed. A spore with two fertile cells probably will result. 12–13. Young spore mother cells with surrounding hyphae. 14. The formation of a 2-celled spore. The hyphae at the base, partly cut off, apparently were growing around to form sterile cells. 15–26. Various stages in the development of spores. It will be noted that each young spore is uninucleate, although there is a dark staining body in 18 that may possibly be a second nucleus. In 17 two nuclei are still visible in one of the sterile cells. In most of the others single nuclei or none are visible.

be surprised if there is still more variability in *Urocystis occulta* than that they observed.

Mycelium and Spore Formation in the Host. Material from smutted plants growing in the greenhouse was killed in Carnoy's solution or in Flemming's weaker solution, embedded, and sectioned in the usual manner. Both cross and longitudinal sections were made through sori in various stages of development. Sections from 5 to 10 μ in thickness were stained in various ways, mostly with the triple stain or Heidenhain's iron-alum haematoxylin. The latter proved by far the most satisfactory, especially with a counter stain of Orange G or eosin.

It is rather difficult to find many vegetative hyphae in which the nuclei can be seen in a considerable number of consecutive cells, because the hyphae are likely to be somewhat gnarled and sporulation may begin soon after a sorus primordium is formed.

Clearly, however, the cells of the vegetative hyphae are usually binucleate, as shown in plate III, 1 to 6. In the early stages of spore formation some of the cells enlarge considerably. They may still be binucleate, even after having attained considerable size (Plate III, 9, 12), or the nuclei may already have fused when the spore mother cells are not very much larger than the other cells (Plate III, 5, 6, 10, 13). By the time cells have begun to look like spores, however, they are virtually universally uninucleate. The nucleus is rather large and a nucleolus is usually clearly visible. By this time, the other cells of the hyphae, except those that develop into sterile cells, are in process of disintegration. The sterile cells are formed from hyphal branches that grow up around the spore mother cell from neighboring cells. The cells of these branches appear to remain binucleate; they become closely appressed to the enlarging spore mother cells, attach themselves to them, and assume various shapes and sizes; their protoplasm and nuclei disappear; and the walls thicken and become dark, as do those of the spores, and the spore is complete (Plate III, 15 to 26).

DISCUSSION

The cytological phenomena in *Urocystis occulta* are similar to those described by Kniep for *U. anemones* and Rawitscher for *U. violae*, except that there is no rigidly fixed method for the initiation of the dikaryophase. Whenever 2 haploid cells containing nuclei of opposite sex are close together they fuse and the nuclei become paired; and the exact place or method seems of secondary importance. By far the most common method is through some kind of fusion between sporidia, but it may occasionally result from the direct passage of 2 or more nuclei from the promycelium into a sporidium, or, very rarely, from the fusion of uninucleate cells in fairly long hyphae. The various methods of fusion indicate clearly that

in this species, as in the other smut fungi, sex is reduced to very simple terms. The gametes are nothing more than haploid nuclei and there certainly are no special gametangia. Consequently, the concept of sex in *U. occulta* must be pushed back to the bare essentials of the phenomenon. The sexual process is the association and union of two haploid nuclei containing different factors for sex, with the resulting formation of a zygote. The really important fact is that there are different factors in different haploid nuclei. If, in addition, in some fungi, there are different factors for growth and for the formation of morphologically different gametes and gametangia, the sex differences become obvious and maleness or femaleness can be distinguished on the basis of characteristic structures. But this additional evidence of sex is merely an elaboration. As concerns the implications of the sexual process, nothing essential has been added by the formation of sex organs, except structures that enable a recognition of sex differences by morphology instead of behavior. The writers are of the opinion that the phenomena in *U. occulta* support this view. Haploid nuclei become associated whenever opportunity offers, but how and where seem to be of relatively little importance, as is shown by the variability of the method of fusion between cells containing them.

While the haplophase of such species as *Ustilago zeae, Sphacelotheca sorghi,* and others may persist for a long time, saprophytically, by the budding of sporidia, the haplophase in *Urocystis occulta* seems to be restricted principally to the sporidia and the promycelium. Normally, therefore, it would last only a few days in nature, and almost the entire active life of the organism would be passed in the dikaryophase, as the diplophase is restricted to the spore mother cells and chlamydospores.

Those who are still loath to interpret sporidial fusions in the smut fungi as part of a sexual process may be skeptical about the sexual nature of the nuclear phenomena in *Urocystis occulta.* It must be admitted, of course, that there is only circumstantial evidence that the single large nucleus in the very young promycelium is diploid, that one of the several successive divisions is a reduction division, and that the nuclei in the sporidia are haploid. However, the fact that there clearly is an affinity between certain sporidia, resulting in fusions of various kinds, seems strong evidence that their nuclei have different factors for sex. Furthermore, one of the fused sporidia usually sends out a hypha almost immediately after the fusion tube becomes continuous, thus uniting the 2 protoplasts. The combined effect of the 2 nuclei in stimulating vigorous growth, even when still separated by a considerable distance, is noteworthy. Binucleate cells have an ability that uninucleate cells apparently do not have. Finally, the rather distinct pairing of the nuclei in 4-nucleate hyphae seems significant, and, certainly, the fusion of paired nuclei at the time chlamydospores are formed can scarcely be anything but the final act in the sexual processes.

As concerns the pathogenicity of the organism, the principal interest in the sexual process is the degree to which it permits recombination of factors for pathogenicity. This could best be studied by cutting off individual sporidia, growing them in culture, and then mating them in various combinations. However, the opportunity for variation resulting from hybridization in *Urocystis occulta,* in which the sporidia do not seem to become detached from the promycelium and apparently do not bud to any extent, seems far more limited than in *Ustilago zeae* and similar smuts, which produce countless sporidia that may multiply saprophytically and be disseminated widely by the wind. Naturally, the number of genetic factors for pathogenicity will determine the number of possible combinations, but in smuts of the *U. zeae* type there is a condition roughly analogous to cross pollination in higher plants. In *Urocystis occulta,* on the other hand, it is more nearly analogous to self pollination, because, ordinarily, only the haploid derivatives of a single diploid nucleus would become paired, unless further nuclear associations occur in the host plant, where the mycelium is already in the dikaryophase. There is, of course, the possibility of "wider crossing" when sporidia of different promycelia fuse. It is possible, also, that, under certain conditions, haploid propagative bodies may multiply to a limited extent, although the writers have thus far obtained no evidence that this may be true.

Theoretically, therefore, there should be fewer parasitic races in *Urocystis occulta* than in *Ustilago zeae.* Whether this is true has not been determined definitely. The writers have inoculated several varieties of rye with different collections of rye smut but found no clear-cut indications of significant differences in pathogenicity. The tests, however, have not been extensive enough to justify definite conclusions.

SUMMARY

The spores of *Urocystis occulta* germinate readily in about 48 hours, producing typically a promycelium of variable length on which are formed from 2 to 6 sporidia that apparently always remain attached to it.

There is a single nucleus in the young promycelium, but several nuclei are formed as a result of successive divisions.

Normally, one nucleus passes into each sporidium, certain sporidia on the same promycelium or different ones then fuse in various ways, a binucleate hypha grows out from one of the fused sporidia or from the fusion tube, and the sporidium from which the nucleus has migrated degenerates and often collapses, as do the nonfused sporidia. This is the usual method by which the dikaryophase is initiated.

Rarely, cells of fairly long hyphae apparently are uninucleate. They may then fuse with other uninucleate cells.

Occasionally, two or more nuclei pass directly from the promycelium into a sporidium, and the dikaryophase begins without previous sporidial fusions.

The host plant probably is infected by hyphae of the dikaryophase. These hyphae may have many cells, but the protoplasm usually is aggregated in the tip cell. Several days after spores have germinated most of the hyphae contain 2 or 4 nuclei, arranged in 2 pairs. Some hyphae seem to contain 3 nuclei, or, rarely, a single nucleus. The 3-nucleate condition probably is often temporary, due to a slight difference in the time of division of the 2 original nuclei or in the separation of the daughter nuclei.

The hyphal cells in the host plant are predominantly dikaryotic until spore formation begins. Then the nuclei in some of the cells fuse, and these spore-mother cells enlarge rapidly at the expense of the others. In the meantime, hyphal branches, often dikaryotic, grow up around the spore-mother cells, appress and attach themselves to them, and lose their protoplasm, the walls of the fertile and sterile cells thicken, and the spore is formed.

There would seem to be less opportunity for variation resulting from hybridization in *Urocystis occulta* than in many other smut fungi.

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A LEAF, STEM, AND POD SPOT OF PEA CAUSED BY A SPECIES OF CLADOSPORIUM[1]

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INTRODUCTION

In the fall of 1932 growers and packers of market peas in certain coastal areas of California directed attention to an apparently undescribed pod spot of peas, *Pisum sativum* L. Field examinations revealed the occurrence of irregular to roughly circular necrotic spots on the foliage, resulting in distortion, and cankers on the stems as well as dark brown to black scabs of irregular shape, which seriously marred the pods. Young, rapidly growing tissue was most affected. Microscopic examination revealed the consistent association of a Cladosporium with the spots. Culture methods readily effected recovery of the causal fungus, which, successfully used in inoculation trials, is herein described as *Cladosporium pisicolum*, n. sp. Distinctly different from other pea diseases, this is typical of other Cladosporium diseases, notably the Cladosporium spot of cowpea, caused by *Cladosporium vignae* Gardner (5).

Occasional references may be found in the literature associating species of Cladosporium with diseased peas. In 1891 Cugini and Macchiati (1, p. 104) described a pea disease in Italy, which they attributed to *Cladosporium pisi*, n. sp. Affected pods dried prematurely and bore scattered, elevated pustules upon which the fungus sporulated. Curzi and Barbaini (2) in 1926 refuted the earlier work of their countrymen by demonstrating that the pod disease was not of parasitic origin but the result of intumescences incited by such factors as temperature, light, and humidity, and that *C. pisi*, which they conclude is *C. herbarum* Link, follows the intumescences as a secondary factor. The descriptions and illustrations of both Cugini and Macchiati and Curzi and Barbaini bear out the conclusions of the latter and clearly set apart the Italian disease from that to be presently described. *C. myriosporum* E. and D., with minutely echinulate conidia, was reported in pea pods in Canada (4) in 1897. Dowson (3) in England described a new disease of the sweet pea in 1924, caused by *C. album*, n. sp., a fungus morphologically identical with *C. herbarum* but white, giving a mildewed appearance to the leaves. Dowson obtained no infection of Pisum. In the

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[2] The writer gratefully acknowledges the counsel given by Professor Ralph E. Smith and Dr. Max W. Gardner.

United States reference was made to a "black spot of peas caused by *Cladosporium* sp. on the seeds" in Washington by Heald (6, p. 49) in 1930. A preliminary report of a leaf, stem, and pod disease of market peas in California was later made by the writer.[3]

DISTRIBUTION

The writer has observed the disease only in California, although it is not unreasonable to expect its occurrence in other pea areas along the Pacific Coast. It may well be that the black-spot disease referred to by Heald is of the same cause. Of the 4 major market pea areas in California, which include Alameda, Monterey, San Luis Obispo, and Imperial Counties, all but the last have been visited the past year. Among these the disease was encountered in most severe form in Monterey County, especially in the Castroville and Salinas district situated along the ocean shore.

ECONOMIC IMPORTANCE

The blighting of young seedlings, interference with proper vegetative development of the vine, and scabbing of the pods are phases of the disease contributing to economic loss. Observations have shown that young seedlings may be killed when badly infected and others so deformed as to be permanently retarded. During certain periods when environmental factors favor the disease, appreciable quantities of blemished pods must be culled out at the packing sheds, resulting in a reduction of 5 to 10 per cent or more of the picked crop. Blighting of the blossoms and very young pods accounts for further losses in yield.

DESCRIPTION OF SYMPTOMS

Foliage symptoms are manifest as roughly circular or irregular discolored spots, which, in early stages and under humid conditions, become covered with a velvety, smoke-colored sporulation. Later, they appear as necrotic tan or brown spots, involving the thickness of the leaf or stipule and sharply delimited generally by a thin line of dark brown to purple (Fig. 1, B). Spots may occur any place within the leaf edge, or marginally, resulting in rapid necrosis of the affected area, which varies from 1 to 8 millimeters in diameter. Where marginal infection takes place marked distortion accompanies continued growth of the leaf, and when such infection is general the cupped or curled, twisted appearance of the foliage (Fig. 2, A, B) becomes recognizable some distance away. Tearing and

[3] Wood, Jessie I., Neil E. Stevens, and Paul R. Miller. Diseases of plants in the United States in 1932. U. S. Dept. Agr., Bur. Plant Indus., Plant Dis. Reporter Sup. 85. 1933. (p. 53–54)

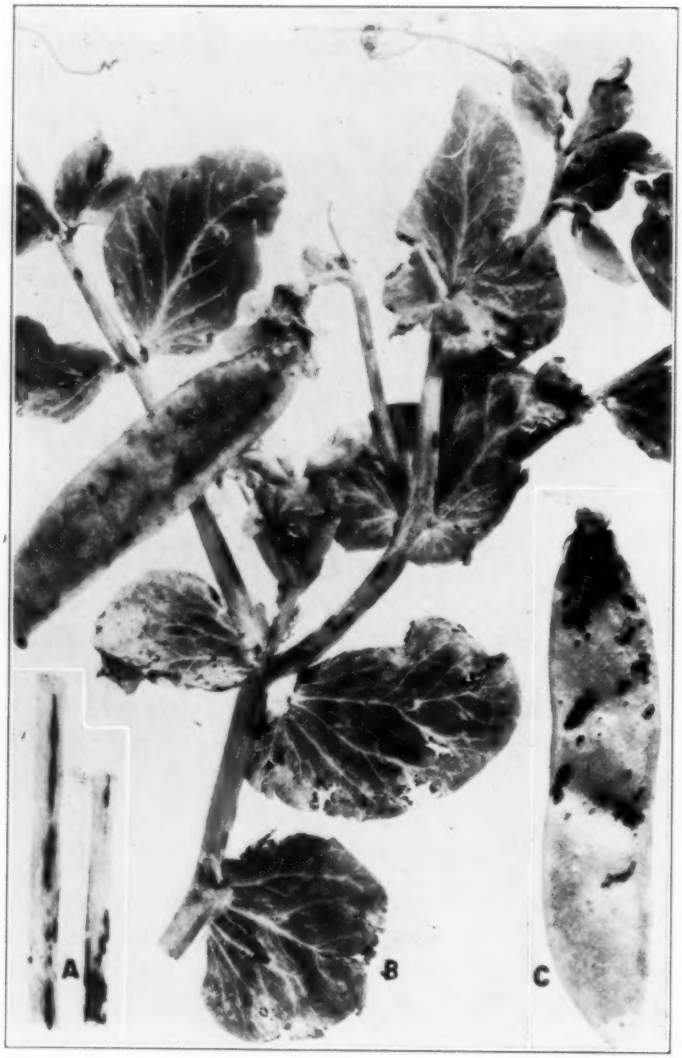

FIG. 1. Symptoms of Cladosporium spot on naturally infected pea. A. Sunken lesions on stems, showing the small lens-shape, crater-like infections as well as long narrow cankers parallel to the vertical axis of the plant. The coalescence of lesions results in irregularly shaped cankers. B. Spots upon the stipules and leaflets occur marginally or interiorly as roughly circular necrotic areas bounded by narrow dark borders. Tearing of the parchment-like lesions gives a tattered, frayed appearance to foliage bearing older infections. Blossom spots do not show well in the photograph, but heavy infection of the attached pod is apparent. These lesions are numerous and mostly small and dark brown, and are sporulating in some cases. C. An advanced pod infection. The black, irregular scabs become sunken in the center with a raised, sharply defined black border. Sporulation on the scabs is abundant with high humidity.

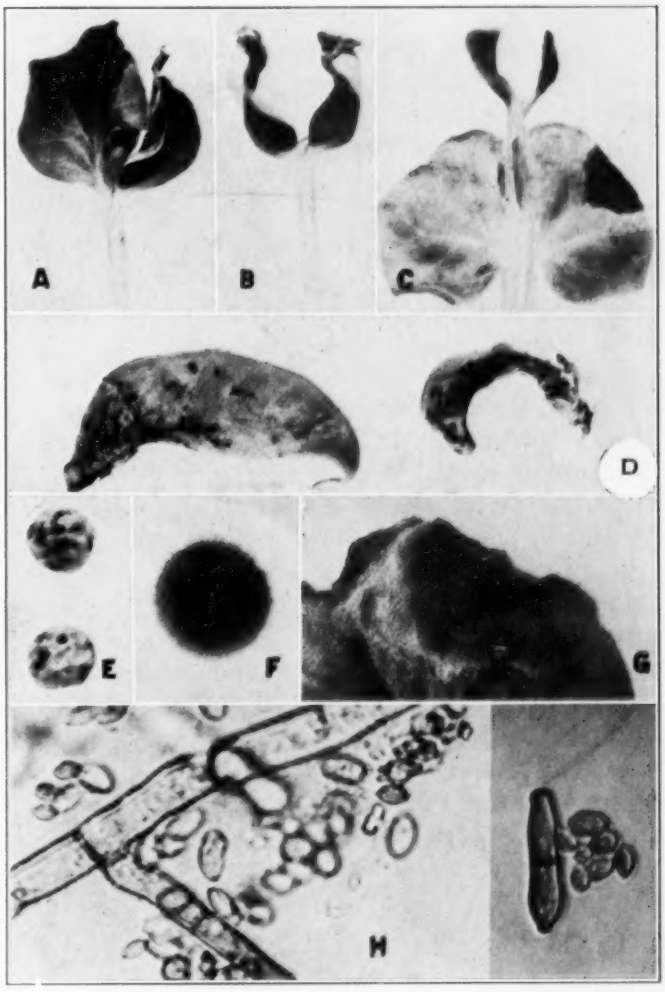

FIG. 2. A–B. Leaves of young pea plants 6 days after atomizing with a spore suspension of *Cladosporium pisicolum*. Sporulation could be observed on the lesions of each leaflet. Note especially the rolled, misshapened appearance, which becomes exaggerated as the unaffected portions of the leaflets continue their development. C. The first stipules and leaflets of a pea seedling grown in infested soil, showing primary infections incurred during emergence. Also typical of primary infections from diseased seed. D. Secondary infections on pea pods from outdoor plants artificially inoculated in the seedling stage 2 months earlier. Observe the severe distortion of the pod on the right. E. Seed naturally infected through pod invasion as the result of secondary spread on plants artificially inoculated 2 months earlier. F. Colony of the pea Cladosporium from a dilution plate on potato-dextrose agar held 8 days at room temperature. G. Sporulation upon a pea leaflet 6 days after inoculation in a moist chamber. ×4. H. Photomicrograph of mycelium and spores, showing the predominant small conidia and one large 2-cell spore. ×900.

fraying of the parchment-like areas of old spots, particularly when marginal, give a tattered appearance to leaves bearing many lesions. These may turn yellow and wither prematurely.

On growing stems, petioles, peduncles, and pedicels, lesions occur first as narrow dark brown or black streaks parallel to the long axis of the affected organ. With continued growth of the part this linear lesion results in a fissure or crack in the tissue. Frequently, the lesions develop into cankers 1 to 4 mm. wide and 1 cm. or more in length, the smaller ones becoming lens-shape (Fig. 1, A, B). With coalescence the cankers extend in irregular patterns over considerable areas and even encircle the affected organ. The lesions are at first slightly sunken. As they enlarge under favorable circumstances, accompanied by continued growth of the part, the lesions deepen and become somewhat crater-like as the result of a raised, ridge-like border. Under moist conditions the crater may be filled with sporulation of the fungus. One sometimes finds severely cankered stems where up to 50 per cent of the surface of an internode may be deeply etched with lesions. The base of the internode where moisture collects under the protecting influence of the stipules is often most severely infected. The well-defined lesions are light to dark brown in the center with a narrow, dark brown to purplish black border. Pedicel lesions sometimes extend over into pod tissue, initiating infection of the fruit, and calyces are frequently infected.

Pod infection often begins beneath dead floral parts, which at first encase and, for a time, adhere to the young pod, upon which the fungus may sporulate. The dark brown or black, very irregular, definitely margined scabs on the pods constitute one of the conspicuous phases of the disease. The scabs, sunken or raised, occurring numerously, as brownish or blackish specks (Fig. 1, B) or sparsely, as large ugly blemishes (Fig. 1, C), give a roughened feel and unsightly appearance to the fruit. Usually measuring 1 to 9 mm. or more in length, the lesions may run together to cover portions of the pod, and, where young ovaries are concerned, cause distortion of the developing structure (Fig. 2, D). Several instances have been encountered where the scab lesions on green pods penetrated the pod wall and discolored 1 or 2 seeds, and more rarely sporulation within the pod cavity has been found, at once suggesting that the fungus may be carried with the seed. Seed infections appear as brown to black spots, which may be scattered over the seed surface as tiny specks or occur as lesions several millimeters in diameter (Fig. 2, E). Small blisters or elevated scabs are produced when immature seed becomes infected, and upon these spots the fungus sporulates (Fig. 3, F). Old seed infections appear as black, roughly circular spots with well-defined borders and may be sunken or crater-like.

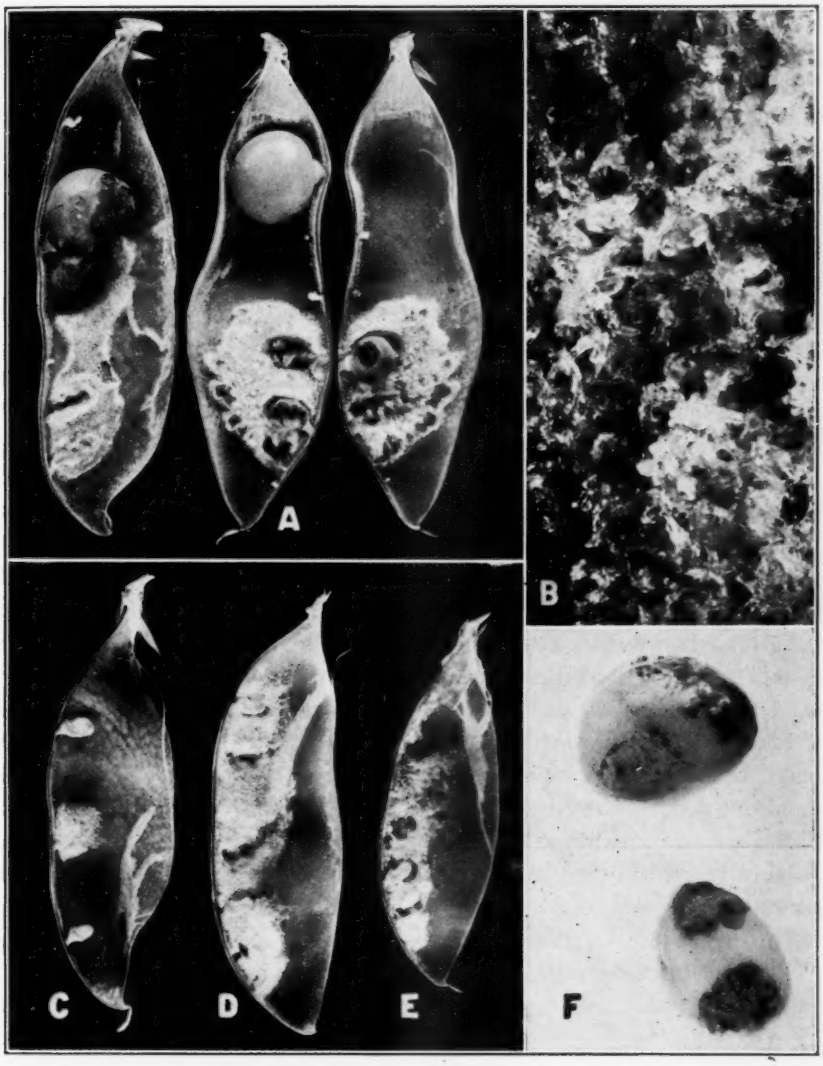

FIG. 3. Results of hypodermic treatments upon attached, growing pea pods. A. Internal Cladosporium infection 12 days after a hypodermic injection of a spore suspension into the pod cavity. Observe the white, felty proliferation containing spots of infection and sporulating lesions upon the seed. B. Proliferated cells of the inner pod membrane, as seen in A. ×22. C–E. Comparison of effects of different hypodermic treatments. C. Air only was forced into the pod. A small area of proliferation has developed about the needle wound. D. Result of injection of a spore suspension as in A. E. Water only was introduced into the pod cavity. The proliferation is much milder and less dense and does not contain the spots of Cladosporium infection obtained in A and D. F. Blistered, lesioned seed from pods treated as in A. ×5.

ETIOLOGY

The fungus, a Cladosporium, was first isolated from scabby pea pods taken from the cull bin of a packing shed near Castroville, in October, 1932. Marginal tissue of each scab, when cultured, yielded an essentially pure culture of the organism. Several of the isolates were selected to inoculate healthy Hundredfold pea plants a month old, growing out-of-doors. The method of inoculation consisted in spraying the vines with a water suspension of fungus spores, by means of a DeVilbiss atomizer. Inoculation was made in the evening, following which bell jars were left over the plants for 14 to 24 hours. Leaf spots appeared upon the inoculated foliage in less than a week, and from these young lesions the Cladosporium was recovered in pure culture and used to similarly inoculate plants of the Laxton's Progress, American Wonder and Hundredfold varieties in the greenhouse. Infection was obtained on each variety in the form of foliage spots, while control plants, atomized with water alone, remained healthy. Inasmuch as 4- and 5-week-old vines had been used in these tests, the next inoculation trial was made on plants only 12 days removed from the date of sowing, averaging at this time 2 inches in height, with the first leaves unfolding. Gardner (5) has shown, in this regard, that cowpeas are most susceptible to *Cladosporium vignae* at the time young leaves are just unfolding. From 12 to 14 young pea seedlings, representing each of several varieties, were placed in a large cloth insect cage, in place of bell jars because of greater convenience and thorougly atomized with a spore suspension derived from a monosporidial culture of the fungus. The experiment was conducted in a greenhouse running at 60° to 70° F. with relatively high humidity; but, as an extra precaution, the plants were given 2 additional sprayings with water only, within the 24 hours following inoculation. Six days later an extensive infection was evident, examples of which are illustrated in figure 2, A, B. Of the 6 seed lots used, Hundredfold, Alderman, Laxton's Progress, Wisconsin Early Sweet, Laxtonian, and Stratagem, all developed infection, ranging from 40 to 100 per cent within the 6 days following inoculation. The degree of infection was moderate to severe. Subsequent controlled experiments, using plants coming into pod, have also proved successful in the artificial inoculation of the fruit by means of an atomized spore suspension, with the production of typical pod scabs.

These first experiments demonstrated the parasitic nature of the Cladosporium in question. Later, pure cultures from monoconidial lines were used, and these also were productive of a rapid and severe disease of all aboveground parts of growing pea plants, the first symptoms appearing in about 3 days, whereas like plants, similarly treated, with the exception that water instead of a spore suspension was sprayed on them, developed into

healthy, normal vines. Attempts to infect cowpea, to which reference will be made later, proved unsuccessful.

MORPHOLOGY AND TAXONOMY

Colonies of the fungus obtained upon dilution plates of potato-dextrose agar (Fig. 2, F) become visible within 2 days, when held at room temperatures. At this age the colonies average 1 mm. in diameter and are already bearing spores, the dusty layer of which gives a vetiver green[4] color to the colonies for the first 2 to 5 days. In less than a week the color becomes modified to deep olive and finally turns olivaceous black with age. Viewed from the underside of the plate the colony assumes a dull greenish black within a few days, which does not appreciably change except to darken somewhat. On potato-dextrose-agar slants the colony, instead of remaining flat on the agar surface, becomes convoluted and wrinkled, and this is also true of plate cultures held at the maximum temperature limits for the fungus. Small dots of grayish white mycelium frequently appear as secondary growth phases on old cultures of the fungus but remain much limited in extent.

The mycelium, which may be relatively hyaline in the interior of infected tissue, where it rapidly ramifies in a much-branched, twisted, septate condition, appears greenish brown or smoke colored, curved but not twisted, and is thicker walled, when on the surface. From this stromatoid mycelium arise the conidiophores, which, with their clusters of conidia, occur abundantly enough to produce a velvety appearance of the infected area (Fig. 2, G). In artificial culture on potato-dextrose agar all the mycelium is dark-colored, especially that part of the colony that is submerged. A dense stand of conidiophores results in a continuous layer of conidia over the surface.

The upright, sometimes slightly crooked, conidiophores are unbranched, dark brown, one to several times septate, and frequently measure 100 μ and more in length. A slight jog or crook may be observed in the region of a septum, marking the previous attachment at that point of a conidial chain. Occasionally, with special precautions in mounting, one may observe 1 to 3 spore clusters, laterally intact at these points, in addition to the terminal one.

The conidia are borne acrogenously in clusters of multibranched spore chains. The basal cells supporting the conidial chains are 2 to 4 times longer than the general run of conidia, cylindrical to cigar-shape, and arise from the sporophore singly or in groups of 2 to 4 but predominantly in groups of 3. On these basal spores are cut off the succeeding conidia,

[4] Ridgway, R. Color standards and color nomenclature. 43 pp.; 53 col. pls. Washington, D. C. 1912.

which diminish in size towards the tips of the chains. The spore chains may consist of 1 to 9 or more conidia and, in addition, may give rise laterally to several branched chains each of which again branches, resulting in a many-chain cluster.

The conidia are light brown, ellipsoid with somewhat pointed ends, or ovoid, and are predominantly continuous, although infrequently one-septate. The larger spores, also brown, are cigar-shape with the base sometimes thicker than the apical portion, continuous or one-septate, occasionally two-septate, and tend to be slightly pedicellate. The smaller spores vary in length from 5.2 to 12.5 μ and 3.7 to 5.5 μ in width, while the larger spores range in length from 12.5 to 23.7 and in width from 4.0 to 5.5 μ. These measurements, based upon the sporulation of week-old lesions, are essentially the same for agar cultures, although the average dimensions of spores produced on lesions tend to be slightly higher.

In examinations of the fungus the writer soon appreciated the close resemblance it bore to the genus Hormodendron, owing to the large clusters of long-branched chains of predominantly continuous conidia borne on tall, erect, conidiophores. However, the presence of occasional bicellular spores (Fig. 2, H) justifies its classification as Cladosporium.

The only description of a Cladosporium parasitic on *Pisum sativum* that has been found in that of Cugini and Macchiati (1) given for the binomial *Cladosporium pisi.* These workers report no stem and foliage blight, an important phase of the disease under discussion, and the pod disease attributed to *C. pisi* coincides neither in description nor by illustration with the pod scab concerned here. The smaller spores, moreover, are described as mostly one-septate, with a constriction at the septum, and measure smaller than the species under consideration. Furthermore, the recent repetition by Curzi and Barbaini (2) of this Italian work leaves little doubt but that the condition dealt with in Italy is of nonparasitic origin and that *C. pisi* is a synonym for *C. herbarum* with no parasitism for *Pisum sativum.*

Cladosporium album, described by Dowson (3) on sweet peas, differs in that it is white and does not infect Pisum. Freedom of the conidia from echinulations distinguishes the present Cladosporium from *C. myriosporum,* reported on peas in Canada (4). Gardner (5) was unable to infect Pisum with *C. vignae,* and inoculations of cowpeas with the Cladosporium from peas have been unsuccessful. In addition, the sporophore and conidial measurements differ, being smaller in the case of *C. vignae,* and the color of colonies in artificial culture does not become so dark with age as does that of the pea Cladosporium. Apparently the Cladosporium concerned here is an undescribed species, for which the binomial, *Cladosporium pisicolum,* n. sp., is proposed.

Cladosporium pisicolum, n. sp.

Colonies on potato-dextrose agar, at first vetiver green, rapidly darkening to deep olive, and finally olivaceous black. Submerged mycelium a dull greenish black. Sporophores numerous, one- to several-septate, unbranched, often slightly bent at the septa, brown, measuring 75 to 225 μ. Conidia borne acrogenously in terminal clusters of branched chains, the spore size diminishing apically. Conidia light brown, the smaller ones ovoid to ellipsoidal, somewhat pointed at the ends, continuous, infrequently one-septate, averaging 4.4×8.7 μ; larger basal ones elongate, almost cylindrical, slightly pedicellate, continuous to one-septate, rarely two-septate, measuring 2 to 3 times longer. Parasitic upon *Pisum sativum* L., producing seedling blight, leaf and stipule spot, slightly sunken lesions on stem, petiole, peduncle, and pedicel, and pod scabs. Leaf spots 1 to 8 mm., tan with a narrow brownish border, roughly circular, interior or marginal. Pod scabs dark brown or black, irregular in shape, sunken or raised, sometimes penetrating the pod wall and invading the seed. The fungus is seed-borne and soil-borne, either source of inoculum infecting seedlings. Found in Monterey and other coastal counties in California.

FUNGUS GROWTH IN RELATION TO TEMPERATURE

The influence of temperature on the linear growth of the fungus was observed by growing the organism on potato-dextrose agar in duplicate at a series of temperatures. Average colony diameters in millimeters after 8 days were as follows for the given Centigrade temperatures: 0 at 3°, 10 at 8.5°, 17 at 11.5°, 29 at 18°, 41 at 22°, 27 at 25°, 19 at 27.5°, 3 at 31°, 0 at 34°. Repetition of the experiment gave no important differences from these results, which establish the minimum for growth between 3 and 8°, the maximum between 31 and 34°, and an optimum near 20 to 22° C.

HOST RANGE

A collection of pea varieties representative of market-pea culture in California was tested with certain other pea varieties and legumes for susceptibility to *Cladosporium pisicolum*. The results are shown in table 1. All plants were grown out-of-doors in glass chambers, which were open during the day but closed at night. Each variety was represented by 6 to 8 plants, and all were inoculated simultaneously with an atomized spore suspension. Repetition of varieties in table 1 indicates seed of different origin. An experiment performed at two different times, gave results of the same order both times.

Owing to the close similarity between the Cladosporium diseases of the pea and the cowpea, a number of varieties of cowpea, found susceptible to

Cladosporium vignae by Gardner (5), were tested[5] in duplicate trials for susceptibility to the pea Cladosporium, in addition to those listed in table 1. This collection included Iron, Large Blackeye, Early Red, New Era, Brabham, Clay, Groit, and, in addition, *Vigna sesquipedalis* Wight, the asparagus bean. No infection was obtained in any instance, although inoculations were made on very young seedlings as well as on unfolding secondary leaves.

All varieties of peas tested showed some degree of susceptibility to the fungus. Most resistant were the Canada field and Alaska types. No variety of cowpea, however, nor other legume tested, was found susceptible to the fungus aside from the exception noted in the case of *Vicia faba* in table 1, although more extensive tests may later reveal other suscepts. The fact that the pea Cladosporium was not found parasitic on cowpea and on sweet pea and that *C. vignae* and *C. album* have been shown not to infect pea indicates that these fungi are not identical with the pea fungus.

SOIL INFESTATION

In certain of the coastal areas of California peas may be found growing almost every month of the year. The fungus has, therefore, no severe climatic conditions to endure nor long periods to withstand in absence of the host. Saprophytic growth of the Cladosporium is abundant upon old pea and other organic refuse and, in this manner, may be carried over in the soil from one planting to another. That carry-over of the fungus in the soil may constitute a particularly potent source of primary infection is illustrated in the following experiences. A fall crop of peas in a field near Watsonville was observed, in October, 1932, to be abundantly infected with the foliage blight of the Cladosporium disease. Near the first of March, 1933, the field was again visited. It had been cultivated but not planted since the removal of the peas in December, and a number of volunteer pea plants 3 to 4 inches high had emerged. Examination revealed that nearly every one of these plants was badly infested with Cladosporium and an occasional plant was killed. The leaves bore large interior and marginal lesions, and stem cankers were extensive and severe. More conclusive evidence, however, was obtained in a greenhouse trial. Diseased pods were incorporated in several pots of soil in October, which were allowed to stand outdoors through the winter. In February they were planted, in the greenhouse, with clean seed and bell jars inverted over the pots upon seedling emergence, to maintain a high humidity. Of 101 resulting plants, 29, or about 29 per cent, showed Cladosporium infection of the primary stipules or leaflets, or both (Fig. 2, C). Where healthy pods had been incorporated in the soil for controls, no infection of seedlings was obtained.

[5] The writer is indebted to Dr. W. J. Morse, Bureau of Plant Industry, U. S. D. A., for the collection of cowpea varieties used in these trials.

TABLE 1.—*Susceptibility of young plants of pea varieties and other legumes to infection by Cladosporium pisicolum*

Species	Variety	Susceptibility[a]	Species	Variety	Susceptibility
Pisum sativum	Morse's 200	++++	Pisum sativum	Improved Stratagem	++
" "	Laxton's Progress	++++	" "	Laxtonian	++
" "	Laxtonian	++++	" "	Hundredfold	++
" "	Morse's Market	+++	" "	Hundredfold	++
" "	Alderman	+++	" "	Senator	++
" "	Alderman	+++	" "	Little Marvel	++
" "	Melting Sugar	+++	" "	Blue Bantam	++
" "	Stratagem	+++	" "	Bruce	++
" "	Laxton's Progress	+++	" "	Resistant Alaska[b]	++
" "	Hundredfold	+++	" "	Alaska	+
" "	Thomas Laxton	+++	" "	Canada Field	+
" "	Perfection	+++			
" "	Wisconsin Early Sweet	+++	Vigna sinensis	Early Buff	0
" "	American Wonder	+++	" "	Columbia	0
" "	Telephone	++	" "	Victor	0
" "	Dwarf Telephone	++	" "	Progressive White	0
" "	Gradus	++	" "	Whippoorwill	0
" "	Giant Butter	++			
			Lathyrus odoratus	Spencer Mixed	0
			Vicia faba	Large seeded	[c]

[a] ++++= very severe, +++= severe, ++= moderate, += slight, 0= no infection.
[b] Resistant to the pea-wilt fungus, *Fusarium orthoceras* Appel and Wr. var. *pisi* Linford.
[c] Moderate infection was obtained in the first trial, conducted under high greenhouse temperatures, but subsequent trials, out-of-doors under cool conditions, yielded no infection.

SEED TRANSMISSION

Brown to black blemishes upon seed from infected pods early suggested that the fungus might be seed-borne. To test this possibility scabby Hundredfold pods were gathered from the cull bin of a pea packer and cured, and the seed harvested by hand. Many of the seed showed discolored spots where contact occurred with the infected pod wall before maturation of the seed. At the end of a 5-month period, during which the seeds were stored at room temperatures, they were planted in steam-sterilizd sand in a clean humidity chamber. Of nearly 100 seed so planted, 5.5 per cent of the resulting seedlings developed Cladosporium spots on the first stipules or leaflets. No other lesions developed and the control plants remained free from disease. In another trial a selected collection of badly spotted seed from infected Alderman and Stratagem pods was planted in the greenhouse near a planting of healthy seed of the same varieties. No attempt was made to provide a humidity other than that prevailing in the greenhouse. Of 13 plants from the infected seed, 9 developed primary lesions on emergence, while no infection appeared on the 15 plants from healthy seed. These and other data demonstrated a high incidence of seedling infection from diseased seed.

That the fungus may be carried within the seed coats as well as on the surface was shown in a trial where spotted seeds from artificially infected pods were surface-sterilized and then allowed to germinate on agar in Petri dishes. Sterilization consisted in dipping the seeds in 85 per cent alcohol and then allowing them to stand in 1–1000 $HgCl_2$ for 5 minutes. After 2 washings in sterile water they were planted in potato-dextrose agar. An occasional seed, on germination in the agar, gave rise to a Cladosporium colony.

In about a week following the appearance of primary infections on seedlings grown from infected seed, secondary infections usually appear in connection with the continued unfolding of new foliage. This is true of plants outdoors or in the greenhouse where watering of the plants, especially when done in the evening, wets the foliage. It is evident that wind and rain are to be considered important agencies in secondary spread of the disease.

ENVIRONMENTAL RELATIONSHIPS

As has been true with most other Cladosporium diseases, a high humidity is favorable, if not essential, to infection. This is particularly true of secondary spread of the pea Cladosporium. Primary infection from either infected seed or soil contamination is apparently initiated prior to emergence in the presence of the soil moisture affecting seed germination. Field infection, occurring late in the development of the plant

and resulting in pod scab, has generally been associated with a period of ocean fogs that for several days may bathe the plants continuously. The ocean breeze bearing the fog also provides for spore dissemination.

Although high humidity seems to be the most important of the environal factors for infection, a moderately cool temperature also is most favorable to the disease and is obtained hand in hand with coastal fogs. In fact, some growers have termed the disease "cold weather blight." The range of temperature most favorable to the disease appears to conform in general with that favorable for the pea plant, roughly, 60 to 70° F., although no controlled experiments on either temperature or humidity have been made. Attempts to inoculate plants held at temperatures much above or below this range have met with only partial success.

Other environal factors are of importance to the extent that they provide for the development of susceptible plant tissue. The young tender tissues are most susceptible, older tissues soon reaching a point where they become resistant to infection, a situation also reported for the cowpea disease. With all organs of the plant it is the appearance of new growth and succulent tissues with which Cladosporium infection is associated, thus confining new infection to the region of the growing points where new leaves are unfolding, to the blossom tissues, and to immature stems and pods.

HYPODERMIC POD INOCULATIONS

In order to observe the effect of the fungus when introduced into the interior of a growing pod, a suspension of Cladosporium spores in sterile distilled water was forced into pea pods of different varieties and ages upon growing greenhouse plants by means of a small hypodermic needle. At the same time a number of pods were treated with the hypodermic similarly, except that in some cases only air, no liquid, was forced into the pod, and in other cases sterile distilled water was injected. A small wound was, therefore, produced in each case but the material discharged was different. The results, observed 12 days later, are as follows:

Of 16 pods injected with air, 14 showed a proliferation of cells of the inner pod membrane for a space about $\frac{1}{4}$ inch in diameter about the wound (Fig. 3, C). No other effects were observed, the remainder of the pod developing normally. Of 40 pods treated with water, 18 showed some proliferation of the pod wall of a mild type spreading some distance from the wound (Fig. 3, E). However, in the cases where the Cladosporium suspension had been injected, 42 out of 47 pods showed pronounced proliferation of the cells of the epithelial lining (Fig. 3, A, D) and, in addition, severe seed infection (Fig. 3, F); the remaining 5 showed seed infection, although the pods appeared to have been too mature at the time of inoculation to react with appreciable proliferation. The fungus could be found

fruiting not only in the patches of proliferations but also abundantly upon the seed (Fig. 3, A). A quantity of these seeds were dried and planted with a resultant very high percentage of seedling infection. The fungus suspension had also, in a few cases, been injected into the upper stem of growing pea plants, resulting in a cracking of the stem and a marked enlargement.

These tests in which the hypodermic needle was used illustrate two points. First, they demonstrate the active parasitism of the fungus, especially upon the green seed; and, second, they indicate that proliferation of the inner pod membrane may be incited by any one of several agencies, namely, a mechanical wound, free moisture, and presence of the fungus Cladosporium. Such proliferation, early described by Sorauer (7, p. 477), has frequently been noted in California coastal areas by the writer to occur in patches upon the interior of pods directly opposite pod infections of, not only Cladosporium, but, also, those of *Ascochyta pisi* and *Peronospora viciae*, and in instances where a developing pod has been punctured or injured while still on the vine and subject to a cool, humid environment.

The proliferation discussed here apparently consists of a hyperplasia of the epithelial lining of the pod cavity, resulting in a mat of hair-like growths (Fig. 3, B), and is not to be confused with the intumescences appearing upon the epicarp as described by Curzi and Barbaini (2). Following stimulation, cells of the inner membrane grow into the pod cavity, divide and redivide, and develop into long, usually curled hairs consisting of several cells, end to end, and containing chloroplasts. It is the production of these hair-like growths of pod tissue that results in the white matted patches, giving a felty appearance to the inside of the pod.

SUMMARY

A leaf, stem, and pod disease of the pea, *Pisum sativum*, is reported as caused by a hitherto undescribed species of Cladosporium, for which the binomial, *Cladosporium pisicolum*, n. sp., is proposed.

The disease has been found most abundant in peas grown for market trade in certain coastal areas of California.

Infection on the foliage appears as necrotic tan spots, roughly circular to irregular, delimited by a narrow line of dark brown. Stem lesions are dark brown to black and of varying shapes. Pod infection results in the formation of dark brown to black scabs of irregular shape with a sharply defined, black line of demarcation outlining them.

Under favorable conditions of high humidity and moderate temperature foliage infections occur within 3 to 7 days following inoculation. Only the young growing tissues are susceptible.

All varieties of *Pisum sativum* tested proved susceptible to infection, although in varying degrees. Cowpea, sweet pea, and the asparagus bean were not found susceptible.

Primary inoculum may be provided by soil infested with vine refuse from a previous crop harboring the fungus or through seed-borne infections of the fungus. Either may operate to cause the blighting of seedlings.

Hair-like proliferations of the inner pod membrane, resulting in white felty patches that extend into the pod cavity, may be induced by presence of the Cladosporium fungus in the pod. Other fungi, free moisture, and even mechanical agencies also appear capable of inciting internal pod proliferation, exclusive of Cladosporium, under the proper conditions.

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CONTROL OF THE VERTICILLIUM WILT OF EGGPLANT[1]

E. F. Guba

(Accepted for publication October 5, 1933)

INTRODUCTION

Eggplant wilt is one of the most serious vegetable diseases in Massachusetts. The losses in yield resulting from it have discouraged many market gardeners from continuing the culture of eggplant (Fig. 1). In view of the importance of the disease in this State, certain fundamental studies of the fungus and related field experiments were undertaken in the hope that some information of practical application might be obtained. The efforts to find feasible control measures have not been entirely successful, but certain information has been obtained that should be of interest.

THE DISEASE

Wilt is prevalent annually in every eggplant field in Massachusetts; frequently the entire planting is a total failure. The disease usually appears in the field after the blossoms have set fruit. It rarely appears among the young plants in the hotbed or plant house. Either individual shoots or the entire plant may be killed, but the infected plants usually grow slowly during the early part of the summer and then revive in August and September. The growth of the fruit stops or is much retarded and the fruit may shrivel. Extensive field observations indicate that the vigor of the plant contributes nothing to its resistance to infection. The darkened color of the wood or vascular tissue, which is the most positive index of diagnosis, extends through the entire system of the plant, including the distal end of the fruit; but efforts of the writer and others (Carpenter (2), Haenseler (3)) to culture the organism from the seeds have been unsuccessful. Frequently, after wilt occurs, other organisms, notably *Sclerotinia sclerotiorum* (Lib.) Mass., aid in the destruction of the plants.

CAUSAL ORGANISM

The causal organism has been designated *Verticillium ovatum* Berk. & Jack. (1), *V. albo-atrum* R. & B., and *V. dahliae* Kleb., without any agreement in the literature, and Rudolph (9) has suggested the name *V. albo-atrum* R. & B. var. *ovatum* B. & J.

The fungus is able to survive indefinitely in cultivated land, due, no doubt, to its ability to live as a saprophyte. At least, no instances of the control of eggplant wilt by long rotations with nonsusceptible crops have

[1] Contribution No. 176 of the Massachusetts Agricultural Experiment Station.

FIG. 1. Verticillium wilt of eggplant.

been observed in Massachusetts or reported elsewhere in the literature. In Massachusetts, the fungus is usually troublesome on okra, tomato, eggplant, potato, chysanthemum, snapdragon, raspberry, blackberry, and maple. It may attack other crop plants and weeds, without, however, always producing the usual wilt symptoms. Aside from those hosts on which the disease is here observed every year, it is occasionally noted on peach, plum, rose, peony, and Japanese barberry. In view of the wide host range and the fact that affected plants wilt with the resulting loss of some or all of their yield, the fungus ranks as one of our most important plant pathogens.

TABLE 1.—*Relation of soil temperature to infection of eggplant with Verticillium*

Soil temperature		Plants infected	
°F.	°F.	*per cent*	*per cent*
55.4	77.	0.0	100.
59.	80.6	25.	50.
62.6	84.2	54.5	50.
66.2	87.8	66.	40.
69.8	91.4	72.7	18.1
73.4	95.	91.6	0.0

The persistence of the fungus in the soil, even in the absence of susceptible plants, and the fact that it is capable of existing as a saprophyte render its control exceedingly difficult. A second planting of eggplant on the same land, following a crop relatively wilt-free, always greatly increases the population of wilted plants and usually results in an eggplant failure. Even in rotations without susceptible crops for many years previous to eggplant, failures from wilt occur.

Observations strongly indicate that cultivated fields everywhere are infested with the fungus and that the soil in the field is the chief source of infection. This conclusion is entertained because the sterilization of the soil in plant beds has not reduced the prevalence of the disease in the field; also, plantings relatively wilt-free always occur even where the same beds are used year after year. According to Haenseler,[2] nearly all of the infection in New Jersey originates in the field, and sterilization of the soil in the plant bed is not considered worth while as a control practice. This has seemed to be the case in Massachusetts.

The fact that the causal organism inhabits the soil and invades the eggplant through its root system precludes any control by spray or dust treatments. For other diseases of a similar nature, control methods have proceeded essentially along the following lines; viz. (a) the development of resistant varieties, (b) soil disinfection or the modification of soil reaction with chemicals, and (c) soil fertilization. These methods were considered in the writer's efforts to find a control for the disease.

VARIETAL REACTION

The problem of developing resistance to eggplant wilt appeared of most interest and of greatest practical value but involved finding resistant individuals or varieties of the host for hybridization purposes. Some unsuccessful efforts in this direction have already been reported by Jagger and Stewart (7) who failed to find any resistance to the Verticillium fungus among a wide collection of strains and varieties of eggplant assembled from different parts of the world. Haenseler (4) reported that the Italian varieties, represented by the long purple types, are slightly resistant and that, in New Jersey, Florida High Bush and New York Improved endure the disease more satisfactorily than does Black Beauty, but none was sufficiently resistant to yield a paying crop on badly infested soils. Chupp[3] from field observations in New York reported Black Beauty as the most susceptible and Florida High Bush apparently resistant. In a more recent report of the study of the reaction of many species, varieties, and strains of

[2] Letter from Dr. C. M. Haenseler, New Jersey Agricultural Experiment Station, New Brunswick, N. J., October 11, 1932.

[3] Chupp, G. U. S. D. A. Plant Disease Rept. Suppl. 45: 144. 1925. May 1, 1926.

eggplant to the wilt fungus, Haenseler (6) noted that none showed special promise for use in developing resistant strains.

In view of the fact that this Verticillium fungus lacks specialization for any particular host or group of related hosts, any possible resistance among varieties of eggplant to the fungus would seemingly be exceedingly limited or entirely lacking. Past accomplishments in the development of crop plants resistant to fungus pathogens appear to have required some degree of host specialization as a prerequisite to success. Vavilov (10), as the result of his extensive studies of the distribution of immunity in connection with the specialization of parasitic fungi, concluded that the more general the field of receptivity to a given fungus, the less likely are there to be found immune or highly resistant varieties in the same species. This fact was clearly demonstrated in the writer's study of the reaction of many types of eggplant and related species assembled from many parts of the world. No evidence of significant differences in their reaction to the pathogen was noted. Since the fungus can infect a wide variety of plants regardless of those characters that define genera, families, and orders of plants, its specialization for certain varieties of a botanical species that differ only in minor and smaller characters is hardly conceivable. The study of the reaction of many varieties of eggplant demonstrates this fact and emphasizes the total lack of any material for selection or for hybridization purposes.

It was observed that some individuals each year failed to show wilt symptoms, but sections of the stems in the basal region of the plants frequently revealed the brown wood characteristic of infection. Some plants were late in their expression of the disease and others even escaped infection. Selection of such stock for resistance extending over a period of several years was, however, totally ineffective.

The early or heavy-fruiting varieties, *e.g.*, New York Purple, showed the first signs of wilt, and late-cropping varieties, *e.g.*, Florida High Bush, always exhibited the disease later in the season. Light-cropping varieties and sterile plants either failed to show wilt or did not exhibit the characteristic symptoms until late in the season. Since the causal fungus is a vascular parasite, it interferes mechanically with the water stream of the plant and consumes water for its own growth. Its presence in the water vessels would obviously induce wilt during periods of maximum transpiration and in times of drought. This would explain the epidemic appearance of the disease when the plants are setting fruit and making their greatest amount of growth and would account for its belated appearance or absence among sterile or light-cropping individuals or varieties. This correlation in the manifestation of the disease with fruit setting and fruiting habits has not been considered by those who have reported degrees of resistance among varieties of eggplant, but it cannot be disregarded in efforts to breed or select for resistance to the disease.

TEMPERATURE RELATIONS

Mycelium was planted on potato-dextrose agar in Erlenmeyer flasks. It was found that growth of Verticillium from eggplant grew at a temperature range of about 50°–95° F. and that the greatest amount of growth occurred at about 78° F. at the end of 72 hours. These results agree fairly well with earlier reports in the literature.

It was found that good growth of eggplant occurred at a soil temperature range of 77°–95° F., with optimum growth at 87°–92° F. The experimental plants were started in sterilized soil and transferred to inoculated soil in soil-temperature tanks in a greenhouse. Twelve plants divided among 4 containers were grown in each soil-temperature compartment. Infection was determined by the characteristic brown vascular ring as revealed in stem

TABLE 2.—*Percentage of wilted eggplants as affected by chemical field treatments in 1930*

Treatment	Rate per acre	Plants wilted		
		July 22	August 10	August 25
	Tons	*per cent*	*per cent*	*per cent*
Copper-Lime dust (20–80)	4	73.6	91.8	97.9
Superphosphate	3¾	74.3	84.5	99.3
Aluminum sulphate	4¾[a]	82.7	82.3	100.0
Winter rye, Fall 1929 seeding		76.6	83.	99.3
No treatment		90.9	90.9	97.2
Inoculated sulphur	6[b]	20.	60.	83.7
Sod 1925–1930. Corn prior to 1925		23.3	65.7	87.6

[a] 1¾ tons applied in the fall of 1929; balance in April, 1930.

[b] 4 tons applied in the spring and fall of 1929; balance in April, 1930.

sections rather than by external symptoms alone. The highest percentage of infected plants was noted at 77° F. At the extreme temperatures, *i.e.*, 59° and 91.4° F., no external symptoms of disease were apparent, although cultures and sections of the main stem revealed the presence of infection. No infection occurred at 55.4° F. and 95° F. According to Ludbrook (8), *Verticillium dahliae* produced marked external disease symptoms at 86° F., but not at 89.6° F. Judging from the number of infected plants at each temperature, the optimum soil temperature for infection appeared to be within the range 73°–80° F. (Table 1).

The Verticillium wilt of eggplant is very prevalent and destructive in northern United States, but, judging from reports, occurrences of this disease on eggplant in the Southern States are rare or lacking. While the disease is definitely epidemic on the eggplant in the cooler regions of this coun-

try, there is nothing about the cardinal-temperature relations of the fungus that might suggest a satisfactory explanation of its restricted geographical distribution on this host.

In a field experiment, in 1929,with paper mulch, it was shown that the higher soil temperatures prevalent under paper caused an earlier appearance and a greater severity of wilt. Soil temperatures under paper at a depth of 2 and 6 inches, respectively, and at similar depths without paper, were recorded daily during June and July. The disease appeared 7 to 10 days earlier and was much more prevalent in the plot grown with paper than in the one without paper. Black mulch paper caused a greater and quicker growth of eggplant during June and early July, and the temperature records indicate that this accelerated growth was correlated with higher soil temperatures. Temperatures at 2 inches under paper were higher and nearer the optimum for infection than at 6 inches.

CHEMICAL APPLICATIONS

The spores of Verticillium are killed with sulphur, copper-lime (20–80), and naphthalene dusts. Aluminum sulphate, in concentrations ranging from 1–100 to 1–400, is lethal. These fungicides were applied to glass slides, and water-drop suspensions of spores were placed on the dry residue. The slides were incubated at 70° F. The lethal action of these chemicals on the spores suggested that some control might be expected from field treatments, although the practical limitations involved were fully realized.

In the season of 1929, naphthalene and copper-lime dusts were added to the hills in the field at planting time. Naphthalene was applied to each hill in quantities of $1\frac{2}{3}$ oz. and 1 oz., and copper-lime dust in quantities of 2 oz. and $1\frac{1}{3}$ oz. It was expected that the treated soil would be disinfected sufficiently to offer some control of wilt, but the results were disappointing. No control of the disease resulted.

Haenseler (5) found that soil pH of 6.0–7.0 was associated with badly wilted fields of eggplant and 4.0–5.0 with relatively small amounts of wilt. According to his studies, the growth of the organism in culture was greatly retarded at pH 4.2–4.5 and at pH 4.0 was extremely poor or lacking. Slight retardation in growth was noted in a pH range of 5.0–5.5, while optimum growth ranged from pH 6.0–8.0. On the other hand, the eggplant was found to tolerate pH 4.2–4.4 without injury. Field tests added support to his findings.

In the summer of 1929 the pH of the soil and the percentage of wilted plants in many fields of eggplant were determined by the writer. In general, a correlation existed between low pH (4.4–5.0) and good control of wilt. Fields with pH 5.0 or above did not show any consistent absence or presence of wilt, and, in view of the fact that high pH was associated with

high percentages of wilted plants, it was inferred that the prevalence of the disease in such fields was due to the lack of an inhibiting soil reaction. The determinations appeared to show that fields naturally acid, as the result of sod culture for several years, provided the most successful control of the disease.

In pure culture with potato-dextrose agar as a medium, growth of Verticillium showed marked retardation at approximately pH 5.0–5.2 when aluminum sulphate was used, and at pH 4.4–4.6 when inoculated sulphur

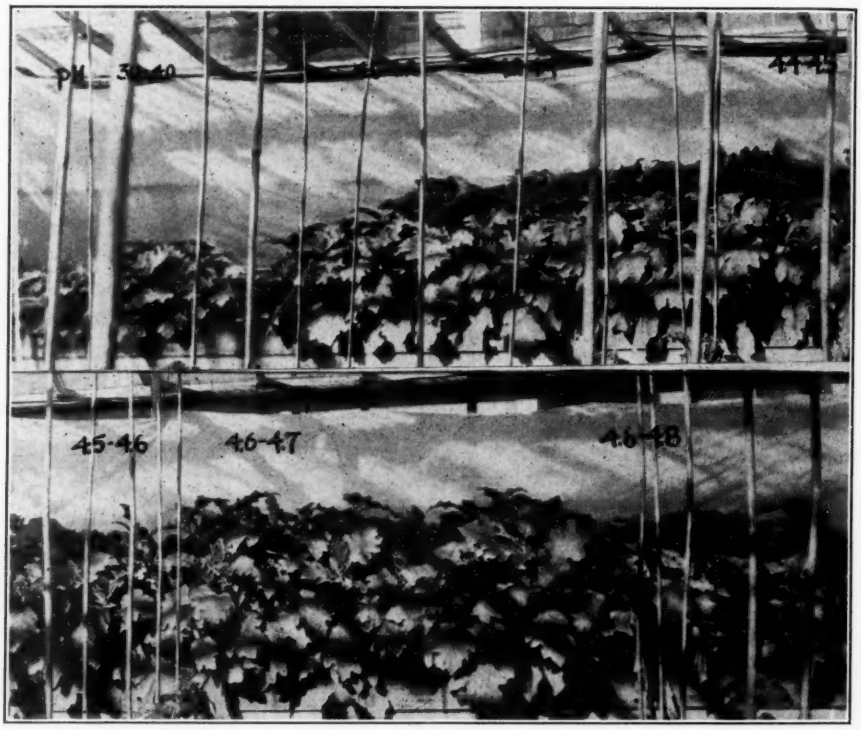

FIG. 2. Eggplant growing in soil at different pH values obtained with inoculated sulphur and showing the extreme low limit of tolerance of eggplant at pH 3.8–4.0 and the control of wilt below pH 4.6–4.8.

was used as the acidifying agent. A pH of 3.6 with inoculated sulphur and 4.0–4.1 with aluminum sulphate completely inhibited growth in culture.

Aluminum sulphate and inoculated sulphur were employed as soil-acidifying agents in a series of pot experiments to determine the lower limits of tolerance of the fungus and the eggplant. The plants were grown in containers holding 1 cubic foot of soil obtained from a naturally contaminated field. Increasing amounts of chemical were added and thoroughly mixed with the soil. The eggplant seedlings used in the tests were grown in

sterilized soil. In the aluminum sulphate series of pots, pH of about 4.7–4.8 impaired the growth of eggplant. No infection occurred below pH 5.0 and no control was obtained above pH 5.5. According to the results a pH range of 4.8–5.5 obtained with aluminum sulphate in the field would be expected to offer some control of the disease without injuring the eggplant. When inoculated sulphur was employed, a pH of 3.7–4.0 and less impaired growth (Fig. 2); excellent control of infection was obtained at 4.0–4.2, and no control at 4.8. Thus, a range of pH 4.0–4.8 in the field with sulphur would be expected to offer control without impairing the growth of the host. Aluminum sulphate gave more consistent control of wilt at the low values than sulphur and, therefore, appeared to be more promising. Undoubtedly, the toxicity of the aluminum, aside from the pH, was an important contributing factor. The result with sulphur confirms the report of Haenseler (5), and better agreement could not be expected. Such narrow ranges in pH would be exceedingly difficult to obtain in practice, however, and likely to be attended either with no control of the disease or with injury to the plants sufficient to render them unproductive.

In April, 1929, a plot of land on which eggplant had been grown for 3 years previously was treated with sulphur at the rate of a ton per acre. The soil of the nontreated plot showed a pH of 6.2. In July the treated plot had a pH of 5.5 and in September of 4.8. No control of wilt was obtained during the crop season of 1929, due apparently to the fact that at planting time the soil was insufficiently acid to exert any appreciable inhibiting effect upon the causal organism.

Eggplant was again grown on these plots in 1930. Comparisons were also made of the effect of different chemicals (Table 2). Copper-lime dust (20–80) was applied at the rate of 4 tons per acre, and superphosphate at the rate of $3\frac{3}{4}$ tons per acre in April, 1930. A heavy crop of winter rye was plowed under in early May and dressings of aluminum sulphate and inoculated sulphur supplemented applications of the previous fall and the spring of 1929. Another plot in an adjacent field was in sod from 1925 to 1930

TABLE 3.—*Percentage of wilted eggplants and corresponding field treatments and soil pH values, July 20, 1931*

Material	pH value	Wilted plants	Plant growth
		per cent	
Aluminum sulphate	5.0–5.6	65	Good
Fall 1929, rye cover crop	5.8–6.2	73	"
No treatment	5.0–5.4	63	"
Inoculated sulphur plot A	3.8–4.3	22	Poor
Inoculated sulphur plot B	3.1–3.9	0	Very poor

and in corn for several years prior to this period. Poor growth and marked dwarfing, but a delay in the appearance of the disease, prevailed in the sulphur plot, and many of the plants died. No benefit was shown from turning under a thick stand of winter rye. Eggplant after sod showed the best growth. In this plot the symptoms of the disease were delayed and the yield was best in spite of the high percentage of infection. In the other plots wilt was general and no benefit resulted from the treatments.

These same plots were seeded to winter rye in the fall of 1930. Growth of the rye was completely inhibited in sulphur plot B and in sulphur plot A there was but a scant growth of it. In the other plots the rye made an even, strong growth. Wilt was well controlled in sulphur plot A, but the reaction of the soil seriously affected the growth of eggplant. In sulphur plot B no infected plants were detected, but growth was extremely poor (Table 3).

Another attempt to control wilt with sulphur and aluminum sulphate in the crop season of 1931 proved unsatisfactory. Applications of inoculated sulphur and aluminum sulphate in April, at the rate of a ton per acre, failed to control the wilt.

Haenseler (5) reported that after 4 years of sulphur treatments and after at least 2 years of extremely acid soil, where the pH was lowered to 4.2, 4.4, and 4.8, as great as 90 per cent, 90 per cent, and 100 per cent of the plants, respectively, showed wilt, although the advent of the disease was much delayed. It may be concluded that artificial soil acidification is not a practical means of control.

Our results, however, indicate the lack of need of lime for eggplant and the desirability of selecting fields with extremely low pH values. Observations and the experience of growers also indicate the undesirability of cropping twice in succession to eggplant, regardless of the soil reaction. Planting each year on new areas of old sod land that is naturally acid has offered the most economical and effective method of combating eggplant wilt.

SUMMARY

No varieties of eggplant showing any degree of resistance to the Verticillium-wilt pathogen have been found.

The fungus is not seed-borne.

The minimum prevalence of wilt was associated with culture on old sod land of pH values below 5.0. Planting each year on new areas of old sod land appears to be the only means of control.

In the greenhouse no infection occurred at soil pH values below 5.0 when aluminum sulphate was used as an acidifying agent, and only small amounts developed at a pH of 4.0–4.2 when inoculated sulphur was employed. Aluminum sulphate gave more consistent control of wilt at the low values than sulphur.

Field applications of aluminum sulphate and sulphur are of no practical value.

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ROOT-KNOT AND OTHER NEMATODES ATTACKING RICE AND SOME ASSOCIATED WEEDS[1]

G. STEINER

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THE NEMIC PARASITES OF THE RICE PLANT PREVIOUSLY REPORTED

The rice plant is subject to a number of nemic diseases. As early as 1902 Breda de Haan (1) described a disease of the rice plant from Java called "omo mentèk" or "omo bambang," which he thought was caused by *Tylenchus oryzae* Breda de Haan 1902[2], the rice nematode, a relationship later disclaimed by the same author (2). In 1913 and 1919 Butler reported another disease of the rice plant from India, with *T. angustus* Butler 1913 as its causative agent (3 and 4). The present paper adds to these two nemic enemies of rice two new ones, caused by widespread and very pathogenic parasites, *Heterodera marioni* (Cornu 1879) Goodey 1932, the root-knot nematode, and *T. pratensis* deMan 1880, the meadow nematode, together with a few other species whose pathological significance is not known or that must be considered parasites of secondary character.

Breda de Haan mentioned a species of Dorylaimus and two species of Cephalobus (one of about 0.9 mm. length and 0.017 mm. width and another 0.45 mm. by 0.021 mm.). The Dorylaimus he reported as rare and as occurring only in parts already beginning to decay. The two Cephalobi may be identical with the two species (*Cephalobus elongatus* deMan 1880 and *C. persegnis* Bastian 1865), which were observed by the present writer in rice-plant roots from Arkansas associated with the forms listed on p. 917.

In 1931 Imamura (6) published an extensive study of the nemic fauna of the soil of a paddy field in Japan, without, however, considering the parasites of the rice plant itself or referring to any relationship between the nemic soil fauna and the plants. Of the numerous species recorded by this Japanese investigator, only two, *Cephalobus elongatus* and *C. persegnis*, were found also by the present writer, as stated above, in the roots of the rice plant.

The parasitic nematode fauna of the rice plant as known at present, therefore, comprises the following forms:

[1] The material for the present study is that referred to by Dr. E. C. Tullis in a paper published in this issue of PHYTOPATHOLOGY. The illustrations were prepared by Mrs. Josephine F. Danforth; technical assistance was given by Miss Edna M. Buhrer, Mrs. Civella A. Brooks, and Mrs. Florence M. Albin, all of this Division.

[2] The generic name Anguillulina has now replaced Tylenchus in general usage. See Baylis and Daubney: A Synopsis of the Families and Genera of Nematoda. London. 1926.

Tylenchus oryzae Breda de Haan 1902
Tylenchus angustus Butler 1913
Tylenchus pratensis deMan 1880
Heterodera marioni (Cornu 1879) Goodey 1932
Aphelenchoides parietinus (Bastian 1865) Steiner 1932
Cephalobus elongatus deMan 1880
Cephalobus persegnis Bastian 1865
Acrobeloides bütschlii (deMan 1884) Thorne 1925
Acrobeloides cornis Thorne 1925

Tylenchus oryzae is insufficiently described; no figures are available. Goodey (5) places it under the species *inquirendae.* A restudy of the species and also of its relationship to the rice plant is needed. In its mode of living in the roots of the host it most closely resembles *T. pratensis,* but the morphological characters, scantily given by its author, prove the form to be different. The spear is said to be knobbed, the median bulb well developed (16 μ wide), with the excretory pore opening ventrad and slightly back of it (75 μ behind anterior end). The tail is described as more or less pointed, ending sometimes with a spine-like, often eccentrically placed, terminus. The average length of the female is given as 1.5 mm. by a width of 0.043 mm., its maximum length as 1.82 mm.; position of vulva as at 63.5 per cent of the total length, that of the anus as at 93.7 per cent; body width at median bulb as 42 μ; spear 19 μ and its knobs 3.3 μ; the oval-shape eggs 102 μ × 26 μ. The male is said to be slightly smaller than the female and the freshly hatched larva to measure 0.5 mm. by 0.01 mm. width.

Tylenchus angustus, in contrast to the aforementioned form, is a well-characterized species, and its parasitic relationship to the rice plant on which it produces a disease of stems and leaves called "ufra" is beyond doubt. For its description we refer to the two papers by Butler.

OBSERVATIONS ON THE DISTRIBUTION OF THE VARIOUS NEMATODE SPECIES IN AN INFESTED RICE ROOT

Only a few roots of plants from the infested rice field were at our disposal, but there was more material grown under greenhouse conditions in flats of root-knot-infested tobacco soil. In the original plants from the rice field only the root-knot nematode was found. The plants from the experimental flats, however, harbored a variety of nemic forms, of which, in addition to the root-knot species, *Tylenchus pratensis* must be considered as a very serious pest. Since in this latter instance the inoculum came from root-knot-infested tobacco roots and soil around them, it is assumed that all species found on the roots from the experimental flats had previously been

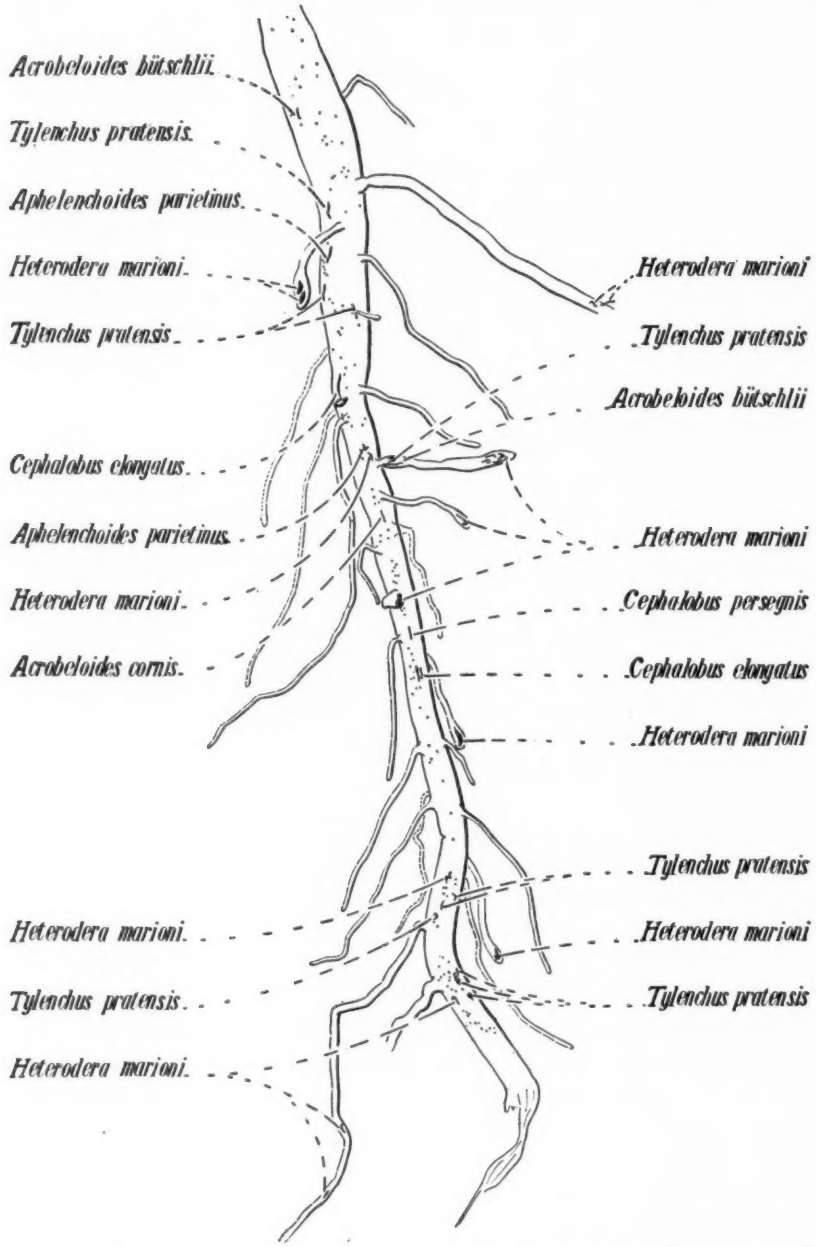

FIG. 1. Portion of root of a 9-day-old rice seedling showing distribution of the various nemic parasites through the roots. The top portion of the root is flattened. All nematodes infesting the roots are drawn in their natural location and position, and as nearly as possible to the same scale as the root itself. ×8.

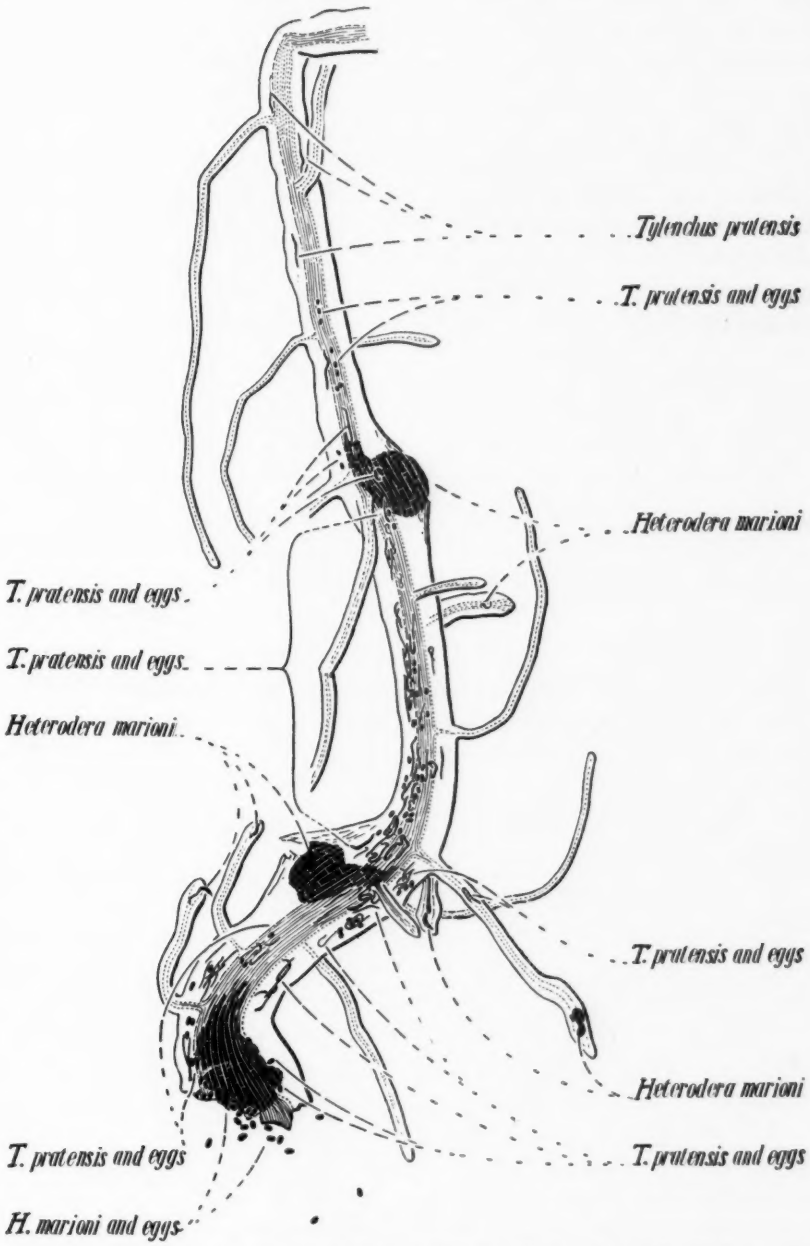

FIG. 2. Piece of a 5-week-old Blue Rose rice seedling root showing a heavy mixed infestation by the root-knot nematode and the meadow nematode (*Tylenchus pratensis*). ×15.

feeding on tobacco plants. As to the significance of *Tylenchus pratensis* as a disease agent in tobacco roots, we refer to a note by the present author (8) and another by Lehman (7). Rice plant seedlings have very transparent roots. Nematodes parasitizing them can be seen clearly without special technique.

To show the location and distribution of these nemas through the roots, a piece of root, about 21 mm. long, is sketched in figure 1. Each nema found there is drawn at the location where observed, in about the same scale as the root itself. The black spots represent chytrids. Eggs were not present in this root portion. The total of nemic specimens observed was 37, belonging to the various species in the following quotas:

Heterodera marioni	18
Tylenchus pratensis	11
Aphelenchoides parietinus	2
Cephalobus elongatus	2
Cephalobus persegnis	1
Acrobeloides bütschlii	2
Acrobeloides cornis	1
	37

The distribution of the various forms through the roots shows that *Heterodera marioni* has a preference for the root tips. This form also is the only one to enter the vascular tissues; all other species remain in the parenchyma of the cortex. *Tylenchus pratensis* has a tendency to form nests, that is, to heap together. This tendency is more plainly seen in figure 2.

THE ROOT-KNOT NEMATODE IN THE RICE ROOTS

It is evident that the rice plant suffers more than numerous other hosts from an attack by this nematode for the following reason: As may be seen from figures 2, C and 3, A in Tullis' paper, the parasitized root tips go blind, and, whereas other plants may counteract and repair the effects of the parasite through additional tissue growth, rice-plant roots exhibit only a reduced faculty for such repair. A comparison of figures 2 and 3, *a* illustrates best the two situations. Figure 2 plainly shows that the enlarging female body pushes the parenchyma of the root cortex aside until the nematode almost reaches the surface or actually breaks through the root epidermis. Thus, through lack of growth of reinforcing tissues, a weak spot develops at which, under tension, the root will break, as the figure so well demonstrates. On the other hand, it is plain that such a situation also is favorable to additional infection by fungi, bacteria, etc., and so, to regular decay, and in-

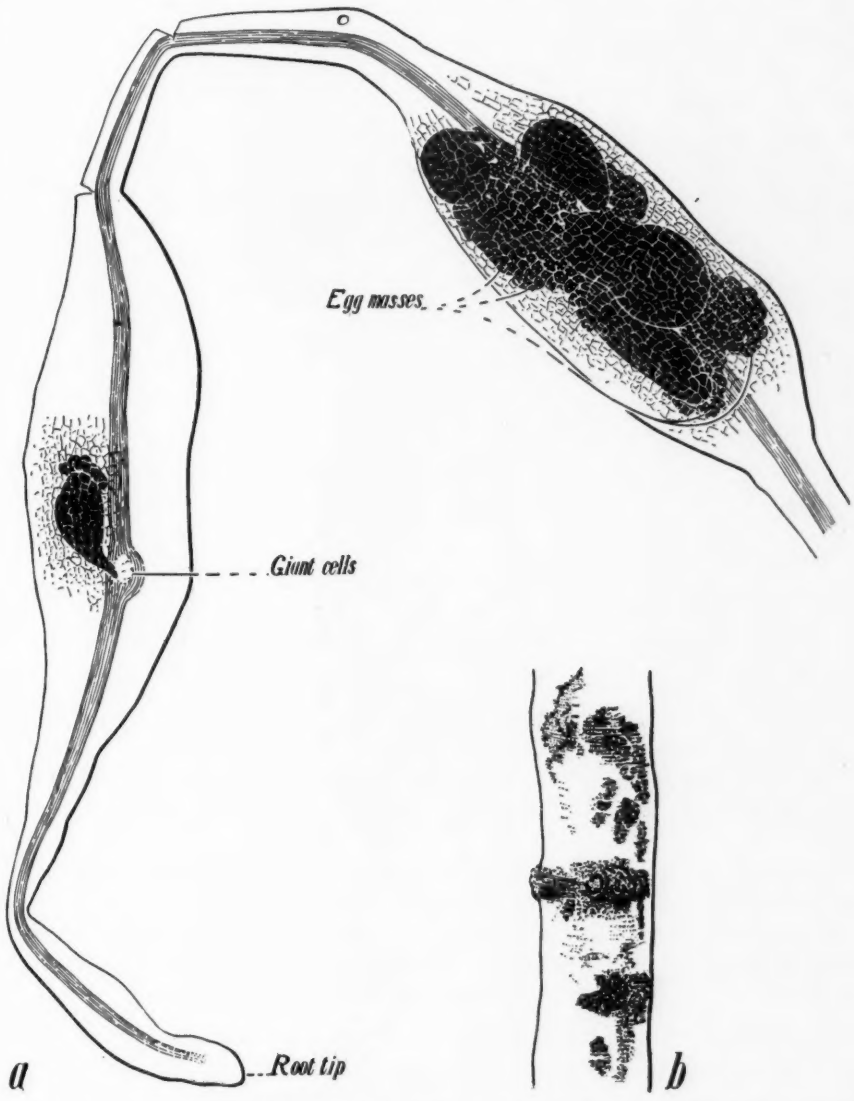

FIG. 3. *a.* Piece of *Echinochloa crusgalli* root infested with *Heterodera marioni.* ×38. *b.* Lesions on a piece of root of *Amaranthus spinosus* harboring *Cephalobus longicaudatus.* ×7.

creases the pathogenicity of the nema, especially as compared with the case of figure 3, *a*. Occasionally specimens are seen that protrude from a root when still in a larval stage (Fig. 4, *b*). In this case the root apparently did not have strength enough or the necessary regenerative power even to grow

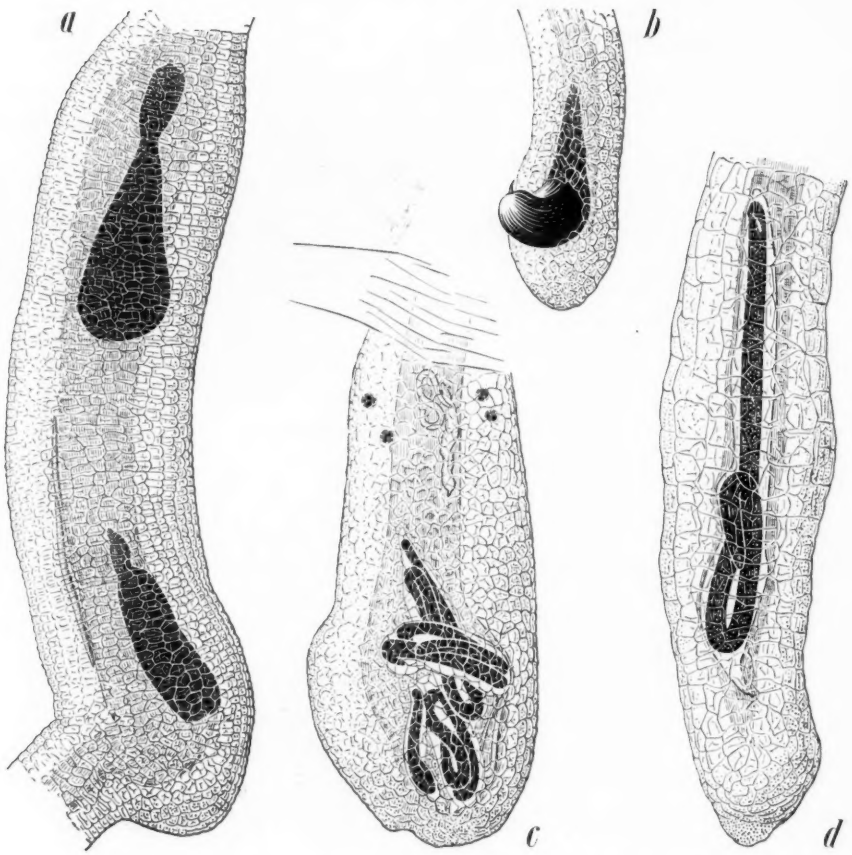

FIG. 4. Root tips of 9-day-old rice seedlings infested with *Heterodera marioni*. *a*. With side root on opposite side of original infestation, which apparently caused the primary root tip to go blind. *b*. Showing larva of the nema partly protruding from the root. *c*. Infestation with seven *H. marioni* specimens, four of which are larval males. *d*. With infestation by a single, but large, larval male. *a*, *b*, *c* ×73; *d* ×94.

around this larva. In the rice plant the root-knot nematode takes the usual position within the root, penetrating the vascular tissue with its head and remaining with the posterior portion of the body in the root cortex. The number of the giant cells is 5 to 6, an unusually large number, but the individual cell is rather small. The giant cells, as far as seen, are always transformed plerome cells. If the number of the latter be increased at all, the increase is certainly not very great (Fig. 5).

As usual, the main point of entrance of the nematodes into the root system of the host is the root tip. It is perhaps because of this that almost all specimens have in the tissue a centripetal position, with the head end toward

the plant. Rarely are specimens seen in the centrifugal position, with the head toward the root tip. This is remarkable because, in *Echinochloa crus-galli* roots (Fig. 3, *a*) this latter inverse position of the parasite seems to be the rule.

The root-knot galls on the rice plant are, judging from the present material, mostly terminal galls, the root tips apparently being checked in further growth by the infestation and thus becoming blind. Of special interest in this connection is the case shown in figure 4, *a*. A comparatively strong

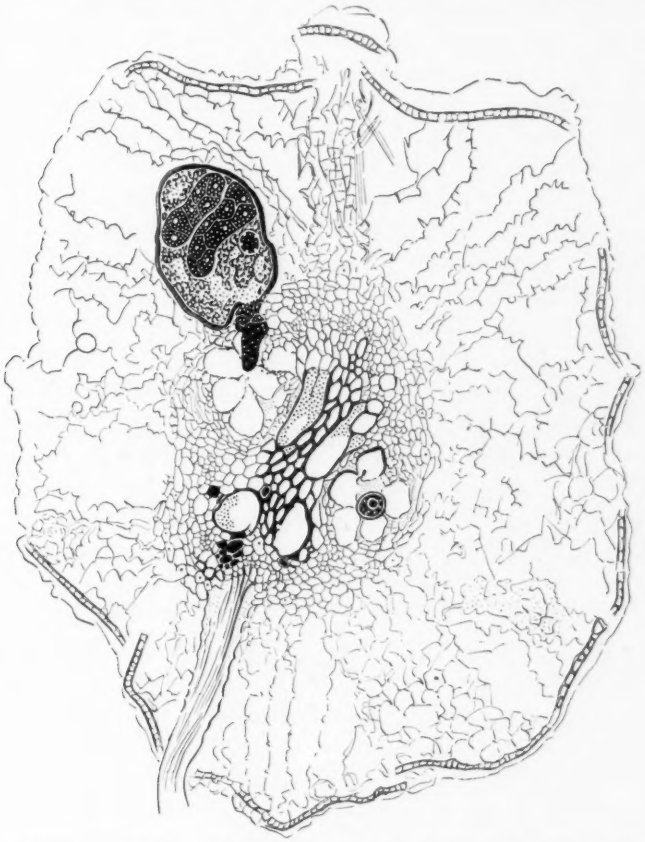

FIG. 5. Cross section through a partly macerated rice root showing the giant cells and the position of the root-knot nema in the root tissue. ×108.

root was attacked by 2 nemas that fixed themselves behind each other on the same side of the vascular cylinder. The primary root tip was thus checked and went blind. However, since the vascular cylinder of the other side was not interfered with and remained strong enough, it grew on and developed a lateral root slightly subterminal to the primary root tip. The figure also shows that the infested root portion was entered again by young larval

specimens that moved to the unaffected side of the root but had not yet fixed themselves in the vascular tissue.

Figures 1 (of Tullis' paper) and 4, *c* (of this), raise the question as to what number of root-knot nematode larvae may enter a given root portion and settle in it. Is space the only deciding factor or are food and other physiological conditions also determining points? Figure 4, *c* shows a root tip with a total of 7 nemas, 4 of which are almost full-grown males. This root is from a 9-day-old seedling, taken from the soil, washed, and sent from Arkansas to Washington. While this handling in all instances killed the female larvae throughout the roots, male larvae apparently developed further, in spite of the complete suspension of growth of the host and the resultant check in the flow of the nutritive sap. This seems to indicate that the males, during their metamorphosis (if such a term may be applied to the last moult of the root-knot nematode male), do not take food and that food shortage may induce the larvae of a given age to develop into males (compare Jocelyn Tyler (10)). The case of figure 4, *d*, too, supports this latter view. It is of interest that all the male specimens within their moult took an inverse position, the tail end of the male being in the head portion of the larval moulting case. Is this accidental or the result of special conditions? Figure 4, *d*, as compared with figure 4, *c*, also shows that the size of the male varies, partly because of the amount of available food, which of course is less in highly infested root portions. From the present observation it is apparent that in rice roots the time needed for the development of the larva from the moment it fixes itself in the tissue to the egg-producing stage is about 4 weeks. The adult female measures about 0.560 mm. in length and 0.536 mm. in width. The size of the eggs was 42 $\mu \times 96.6$ μ–98.7 μ.

TYLENCHUS PRATENSIS AS A PARASITE IN RICE ROOTS

It is the first time that this nema is recorded as attacking rice roots. In this case the infestation apparently originated from tobacco. It is not known if this species would survive and persist in a rice field if under water. *Tylenchus pratensis* is found only in the parenchymal tissues of plant roots and the rice plant is no exception to this rule. In the present case only females and larvae were observed. Figure 6, *b* shows a group of embryonated eggs of apparently the same age deposited close together. Inasmuch as nemas were seen in this region of the root, it is thought that a single female deposited these eggs and afterwards left, according to the vagrant habit of this species. If these eggs had been left to develop, they would have given rise to a "nest" of *Tylenchus pratensis*. The seedling plant in whose roots these eggs were found was 9 days old when taken from the flat. It was cleaned of soil and sent from Arkansas to Washington, D. C., where the material was then fixed. Within this period of time (about 2 weeks) the eggs, which are deposited unsegmented, developed to the embryonate stage (Fig.

6, *b*). Figure 6, *a* shows a female that apparently had produced the 6 eggs. *Tylenchus pratensis* eggs, which measure 20–21 μ by 60–68 μ, are often slightly incurved, especially in certain cleavage stages. Because of their much greater length than width the 4-cell cleavage stage shows the cells still in a serial arrangement (Fig. 6, *c*). In the root these eggs have regularly a more or less axial position. In no instance was an egg seen in a

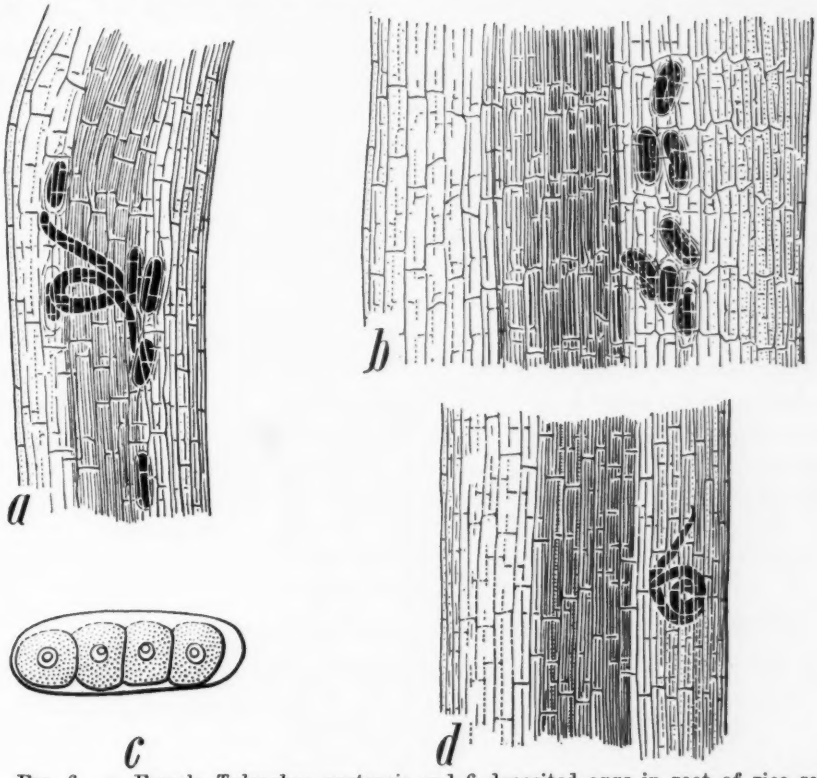

FIG. 6. *a*. Female *Tylenchus pratensis* and 6 deposited eggs in root of rice seedling. *b*. Embryonated eggs of *T. pratensis* in root of rice seedling. *c*. Egg of *T. pratensis* in 4-cell cleavage stage. *d*. *Aphelenchoides parietinus* specimen in healthy root parenchyma of rice seedling. *a*, *b*, *d* ×110; *c* ×470.

strictly transverse position. Whether this is the result of the character of the root cells and the type of tissue or of the mode of deposition remains an open question.

There is a fundamental difference between a parasitism as effected by the root-knot nematode and that by *Tylenchus pratensis* or other forms here mentioned. The former is a sedentary, fixed parasite; the latter are vagrant forms. The root-knot nematode transforms the host tissues through injections. Giant cells—galls—are formed by the host as a reaction to these injections. The giant cells are nectarials—food cells. *Heterodera marioni*

has, therefore, extra oral, perioral digestion. *T. pratensis* is very different in this respect; producing no galls or giant cells, it in no way influences the host tissues to specific reactions. Its digestion is still decidedly intestinal. Apparently, the plasma of the parenchymal cells is ingested by this species, but a direct proof of this is not yet available.

Němec pointed out that the larval Heterodera uses the intercellular spaces to move through the tissues. Apparently, this habit has some relation to the fact that this nematode needs an otherwise healthy root that is able to react, so that the giant cells may be formed. Only a root that is undisturbed in other respects, the tissues of which are unharmed, may do so. It seems, therefore, to be in the interest of the parasite not to harm the tissues more than necessary; hence, apparently, the migration by means of the intercellular spaces. The other parasitic forms here mentioned are quite different in this regard. They force themselves through cell walls and damage or destroy cells and tissues in other ways. These latter nematodes are thus mechanically destructive and, in this respect, different from the root-knot form, which primarily only weakens a plant by extraction of food. Of course, secondarily, additional damage is done to certain hosts by interference with the flow of sap in the vascular system and by breaking up the tissues through the expansion of the parasite's body. (Fig. 2). A parasitism like that of *Heterodera marioni* is less likely to produce general decay than that of *Tylenchus pratensis* and other forms of the vagrant type.

APHELENCHOIDES PARIETINUS AND THE OTHER FORMS OF MINOR SIGNIFICANCE IN RICE SEEDLINGS

Aphelenchoides parietinus, Cephalobus elongatus, C. persegnis, and *Acrobeloides bütschlii* are forms of world-wide distribution. For none has the exact status as a plant pathogen been worked out. Although best regarded as secondary disease agents, it would be wrong to consider them only related to decay, for all these species also are found in healthy tissues, where decay has not yet set in—the situation in the roots of our rice seedlings. The *Aphelenchoides parietinus* sketched in figure 6, *d* is plainly shown within otherwise structurally healthy parenchymal tissue of the cortex. Of course, the root, part of which was sketched, already harbored numerous parasites that interfered with the normal root functions, creating physiologically unhealthy conditions and, subsequently perhaps, an incitement for these secondary parasites. Undoubtedly they had only begun their phase of destruction, the specimens observed being mainly in the larval stage.

Unlike the aforementioned species, *Acrobeloides cornis* probably is of restricted distribution, observed heretofore only in the Rocky Mountain region. The specimen seen agreed with those specimens described by Thorne (9), except that the tail end was more pointed and not so obtuse.

THE ROOT-KNOT GALLS OF ECHINOCHLOA CRUSGALLI

Amaranthus spinosus and *Echinochloa crusgalli* were associated in the field with the infested rice plants. Only the latter of the two weeds was attacked by the root-knot nema. The reaction of this host plant to the parasite differs somewhat from that of the rice plant. The galls are larger and neatly oval or spindle-shape (Fig. 3, *a*). In no instance did the tissue crack to give space to protruding female nematodes. A comparison of infested rice and *E. crusgalli* roots shows the latter better fitted for this parasitism. The roots form "healthier" galls, if this expression be permissible; their tissues are seemingly less interfered with. In the rice roots, as mentioned above, the parenchyma of the cortex appeared unable to make the necessary supplementary growth to form proper galls. In *E. crusgalli* the nemas have most often an inverse position in the root, indicating an entrance by the parasite in a nonterminal section of the root, whereas a "normal" position would result from an infestation by way of the root tip.

In regard to the formation of giant cells in Echinochloa, it may be pointed out that only 3 or 4, rarely 5, are formed. In total preparations of the roots they often show as a light, more transparent spot, immediately surrounding the head end of the nema (Fig. 3, *a*). As may be seen from the same figure, considerable masses of eggs are pressed into the parenchymal tissue of the cortex immediately surrounding the vulva. A gelatinous substance is found around the eggs and the whole mass of both is often larger than the female nema. But an egg sac, such as described by other authors, was not seen.

AMARANTHUS SPINOSUS AND ITS NEMIC PARASITES

Although growing in close association with the root-knot-infested rice and *Echinochloa crusgalli* plants and known to be a host of *Heterodera marioni*, it was rather surprising to find *Amaranthus spinosus* not attacked by this nema. Probably this is a case of a root-knot population with a restricted host range that excludes the amaranth. However, the roots of the present plant harbored various other nemas, some of which are of special interest, as may be seen from the following list:

Rhabditis sp. 4 females; 4 larvae.
Cephalobus striatus Bastian 1865. 1 male; 3 larvae.
Cephalobus elongatus deMan 1880. 1 larva.
Cephalobus longicaudatus Bütschli 1873.
An apparently new genus of the Cephalobinae. 6 males; 3 larvae.
Plectus rhizophilus deMan 1880. 4 larvae.
Tylenchus multicinctus Cobb 1893. 5 larvae.

Tylenchus dubius Bütschli 1873. 1 male; 1 female; 3 larvae.
Tylenchus leptosoma deMan 1880. 1 female.
Aphelenchoides fragariae (Ritzema Bos 1891) Christie 1932. 1 female.
Three Dorylaims belonging to 2 different species.

Aphelenchoides fragariae was seen in a single specimen of undoubted character. Its appearance in these amaranth roots is rather surprising and might be wholly accidental.

Tylenchus multicinctus is a form of wide geographic distribution, especially in tropical and subtropical regions, but little is known as to its exact significance as a plant pathogen. *Tylenchus dubius* also belongs in this category. The Cephalobs were represented by 4 different species, *Cephalobus striatus* and *C. elongatus* being well-known decay associates with decided pathogenic characters of their own. *C. longicaudatus* is a rather rare form. It was found in reddish brown lesions (Fig. 3, *b*) on only one of the amaranth roots. However, its nature as a causative agent of these lesions was not checked by experiment and remains questionable. Another Cephalobus was found that apparently represents a new species and genus and will be described elsewhere.

Plectus rhizophilus and also the Dorylaims mentioned are obscure in their significance in the root tissues where found.

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EXPERIMENTAL PRODUCTION OF CROWN GALL ON OPUNTIA[1]

MICHAEL LEVINE

(Accepted for publication November 13, 1933)

In an earlier report the writer (3) called attention to the fact that young plants of the tree cactus, *Carnegiea gigantea,* respond to inoculations with *Bacterium tumefaciens* by producing typical crown galls comparable to those of the red beet and other garden plants generally used in experimental studies of this disease. It was pointed out that Smith (5) was able to produce a growth in one of four varieties of Opuntia that regressed, a phenomenon common in animal tumors but not reported for plants.

As far as could be determined by a survey of the literature, crown gall on other Cactaceae has been unknown. Brown and Evans (1) report that galls on the giant cactus, *Carnegiea gigantea,* have been known to the senior author for 20 years and have been regarded by him as crown galls. These structures occur on the stem and root of the plant and attain a diameter of 2 or more feet on the stem, and even larger overgrowths have been found on the root. Culture of the crown-gall tissue yielded, according to these authors, a uni-to-bi-flagellate bacterium, measurements of which correspond to those of the crown-gall organism. Unfortunately, the authors fail to give these measurements, for upon them apparently, rests their only evidence that they have isolated the tumor-producing bacterium. Their inoculations have not yet resulted in the development of a single crown gall.

There is evidently no reason to suspect the absence of crown gall in Arizona, as Lockwood's (4) interesting account of the early agricultural activities in this country relates that the Mission Gardens were stocked with fruit trees, common hosts to the crown-gall organism. Proof of the existence of this disease in nature on the Sahuaro has not yet been entirely established.

METHODS AND MATERIALS

The apparent interest in these very unique plants, the Cactaceae, and the inability, up to the present, to produce tumors on the Opuntia species, have led me to repeat inoculations on freshly rooted joints of the same group of Opuntias reported in my previous communciation. (*Opuntia Keyensis, O. Dillenii, O. stricta,* and *O. Linderheimeri* and a large number of plants produced from joints of a species of Floridian Opuntia.)

The methods of inoculation used in these tests were similar to those previously reported, but, the time of the inoculations was changed. As far as the ordinary garden plants, hosts of crown gall, are concerned, it has

[1] Completed with a grant-in-aid made by the Chemical Foundation, N. Y.

been a generally established custom to inoculate them while still actively growing and young; that is, in the early part of the growing season, usually May, June, and early July in the North. In these experiments the date of the inoculation was set for the latter part of July and August up to early September. The joints studied in these experiments were grown in pots sunk in the ground in the experimental garden at Montefiore Hospital.

A very virulent strain of *Bacterium tumefaciens* known in my culture as 6NIS[6] was isolated a number of years ago from a nonsterilized fragment of geranium crown gall, which consisted of an interior and a surface portion of the neoplasm. The inoculations were made in the usual manner by piercing the edge of the joint with a sterile needle immersed in a culture of the organism.

The plants were removed from the garden the latter part of September and kept in a cool room where the temperature never exceeded 20° C. during the winter months, with diffused light on sunny days only. In January of the present year small swellings appeared on the edges of *Opuntia Keyensis*. In June these plants were set out in the garden again, when it was noticed that well-developed crown galls had been formed on a number of inoculated joints of this plant. Portions of these galls were fixed in Bouin's and Flemming's weak solutions. Paraffin sections 5 μ to 10 μ were stained in Heidenhain's iron haematoxylin and Flemming's triple stain. An excellent stain for this tissue consists of an aqueous solution of safranin counter-stained with light green dissolved in oil of cloves. The nuclei in these preparations are ruby red with nucleoli somewhat more deeply stained, while the cytoplasm is pale green and the cell walls become brilliant green. The other Opuntias inoculated have given no results other than development of necrotic areas at the region of bacterial implantation.

OBSERVATIONS

Inoculations of several species of Opuntia listed above with a virulent train of *Bacterium tumefaciens* resulted only in the development of crown galls on the well-rooted joints of *O. Keyensis*. These galls begin to appear 4 to 5 months after inoculation and attain the size of hazelnuts in 6 to 7 months. Figure 1 shows 2 plants approximately a year after inoculation. The galls have grown to the size of a walnut and are smooth and sessile. They are pale greenish yellow with minute brownish to reddish spots on their surface. The galls grow slowly at first, but, like young joints once formed, make very rapid progress in development. At present (July, 1934) they have attained the size of well-formed oranges with no apparent necrosis. The joints of this species of Opuntia are characterized by weak spines and a closer examination of the surface of the gall reveals the presence of reddish brown papillae or soft spine or root-like structures. On

FIG. 1. *Opuntia Keyensis.* ×¼. A. Side view of joint with crown gall on edge of main shoot at the site of inoculation with *Bacterium tumefaciens* on Aug. 4, 1932. Photographed July 27, 1933. B and C. Another plant of the same species showing crown gall. Inoculated on Aug. 4, 1932, and photographed approximately one year later. Microscopic preparations were made principally from plant shown in A.

removing parts of these galls a bark-like coating is formed over the cut surface without any disturbance to the rest of the tumor. These galls have been watched carefully and, at present writing, no mature spines have been observed on the galls, as in the case of *Carnegiea gigantea.*

Microscopic sections of these tumors prove to be of great interest. The crown-gall tissue, as in the Sahuaro, is soft and sections very readily. A

FIG. 2. Section of crown gall showing conglomeration of disoriented parenchymatous and fibrovascular elements. × 50.

photomicrograph of the histological structure of a gall under low magnification is shown in figure 2. Here one recognizes the compact corky structure of the surface consisting of several layers of cells in thickness, which is broken and peeling off; lying below and making up the body of the tumor, is a conglomeration of cells of various sizes. Cross and longitudinal sections of disoriented fibrovascular elements are seen surrounded by parenchymatous tissue. Scattered along the surface of the tumor mass, as

shown in figure 3, are a number of definitely organized apparently normal embryonic structures. In young stages these bodies appear to be globular (Fig. 3) and elongate as they grow older (Fig. 4). These structures in their early stages appear normal, consisting of a growing area made up of small embryonic cells that stain deeply, like the embryonic root tips of other plants. The nuclei are comparatively large, well differentiated, and division

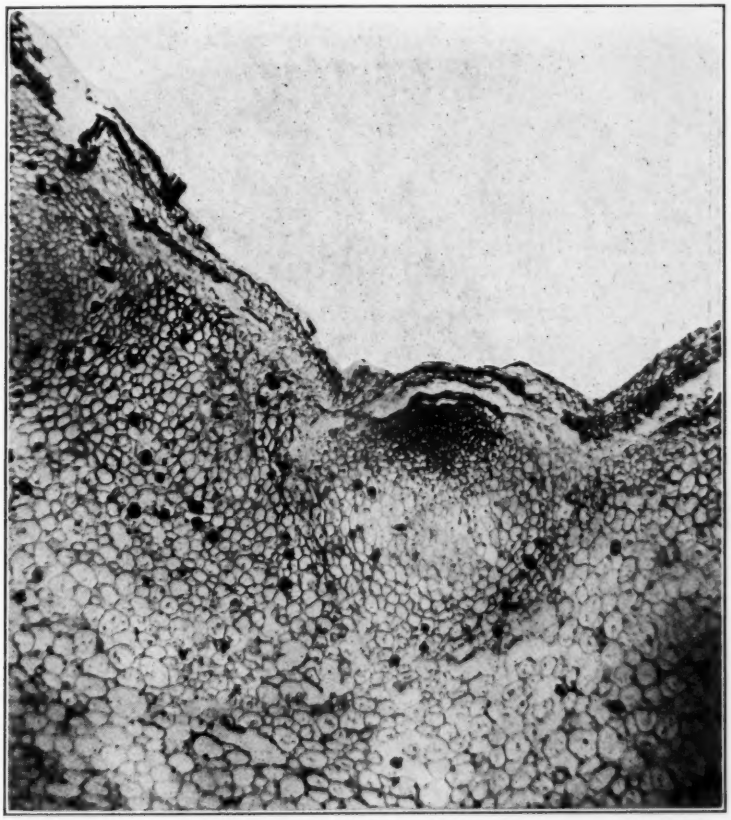

FIG. 3. Section near surface of gall showing highly organized structures imbedded in the crown-gall tissue. Apparently it was developing roots. Note growing point made up of densely staining embryonic cells apparently growing through the cork-like surface of the gall. ×50.

stages are numerous. This growing point lies near the periphery of the tumor, under the epidermis through which it appears to be growing. A middle or stalk portion made of larger cells appears to constitute the region of elongation, while the basal part of this stalk region, farthest away from the surface of the gall, consist of 2 or 3 layers of cells forming a definite wall about this structure. Here the cells are larger and the nuclei are well

differentiated and occupy a central position in the cytoplasm. This tissue shows a moderate number of cell and nuclear division stages. Cross-sections of these embryonic structures below the growing region appear frequently in my preparations (Fig. 5). Surrounding these embryonic structures are large cortical cells that are frequently binucleate. Many cells in this area are filled with aggregates of crystals.

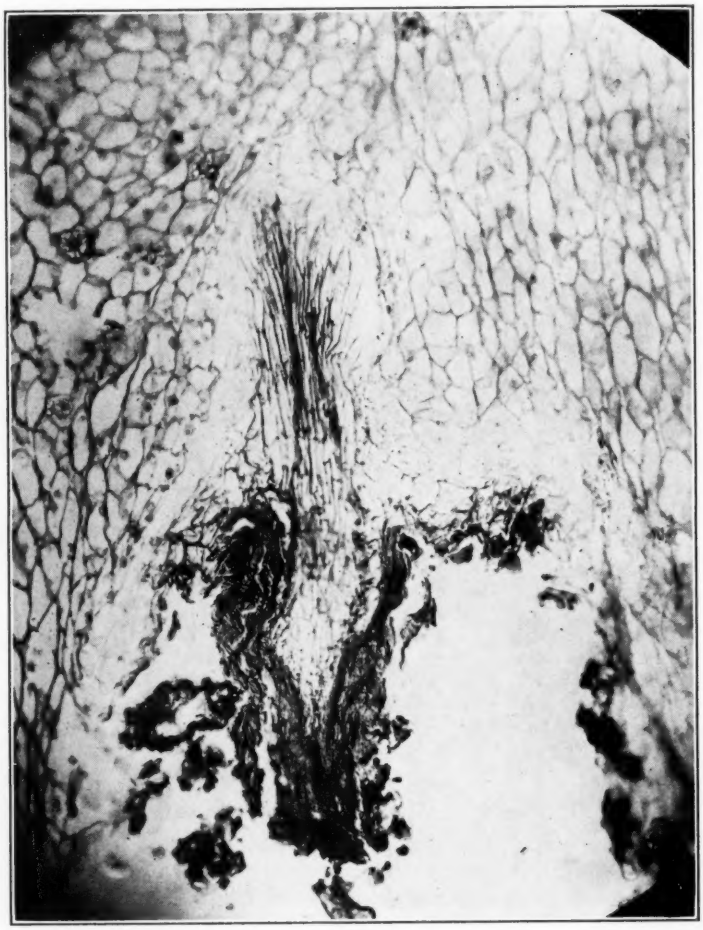

FIG. 4. Abortive young root, necrotised, on surface of crown gall. ×50.

In microscopic sections of some galls I have found aborted multicellular projections that are spine-like and probably represent the later development of roots described above. These structures (Fig. 4) arise in the parenchymatous tissue of the gall. Fresh and active fibrovascular elements appear to grow into these projections, while the epidermis and cortex

are dry and apparently dead. The tissue surrounding this abortive root consists of cortical cells; cells filled with aggregates of crystals are scattered through the tissue.

Clusters of embryonic cells and imperfectly organized embryonic roots also appear in the mass of parenchymatous tissue that constitutes the major part of the gall. Some of the former cells appear in the interior of the gall and ultimately give rise, by differentiation, to strands of fibrovascular elements. The latter appear near the periphery of the tumor under the surface of fragmentary masses of epidermal and cork cells. Their ultimate

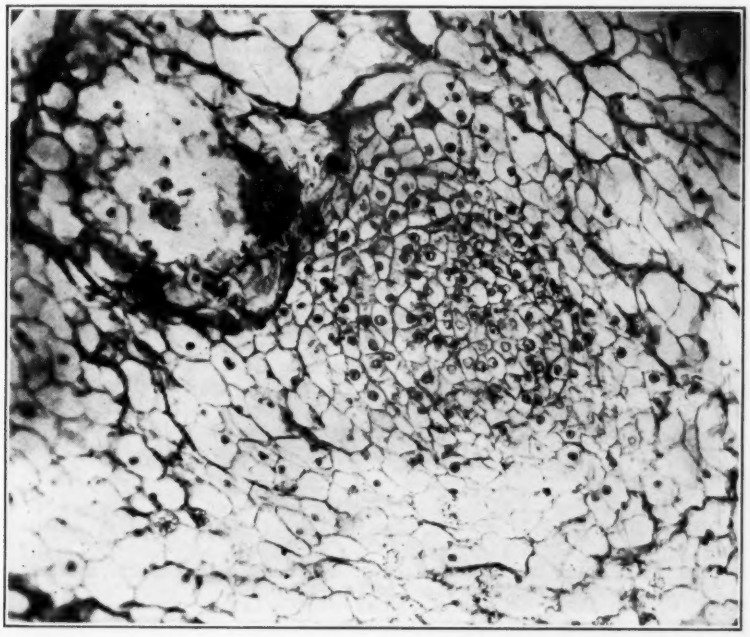

FIG. 5. Cross-section of an embryonic root imbedded in the crown gall shown in figure 3; well-differentiated nuclei are present. ×100.

fate has not been followed, but it is likely they break down early in the aging of the gall. The parenchymatous or cortical cells became large and stained beautifully with the Flemming's triple stain. Binucleate cells appear in large numbers in the tissue under the epidermis studied.

It appears that the tumors of the Opuntia are not simple growths as shown in the small nodular neoplasia described for the beet (2) and Sahuaro but represent growths with a number of growing centers in the interior of the gall. The activity of these centers brings the old differentiated tissue of the gall to the surface. Growth in these tumors appears to be internal and peripheral.

Preparations for microscopic study were made of these plants so that the host and crown-gall tissue appear in the same section. Such a preparations is represented by figure 6. The small parenchymatous cells, to the left of the slightly elongated cells, represent the crown-gall tissue, while to the right is the host tissue with larger cortical cells. The large cells filled with stratified layers of mucilage or slime, similar to those described for the tree cactus (3), are found only in the host tissue. Zones of deeply stained material appear to surround these cells. The small black bodies in both tissues are cells filled with aggregates of crystals.

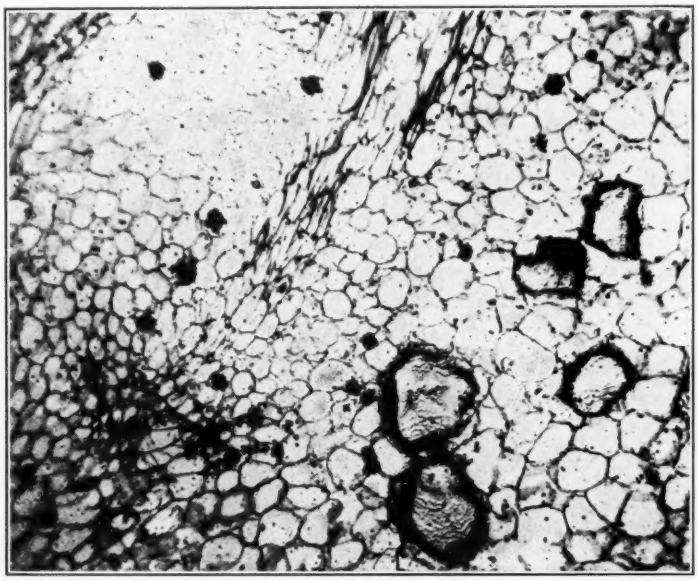

FIG. 6. Section through the host and crown-gall tissue separated by a strand of elongated parenchymtous cells. Note absence of mucilage or slime cells in crown-gall tissue on left of strand. Black bodies in both tissues are cells containing aggregates of crystals. ×100.

SUMMARY

Crown gall on *Opuntia Keyensis* has been induced by inoculation with a virulent strain of *Bacterium tumefaciens*. Under conditions not yet clear *O. Keyensis* inoculated in the late summer and kept at moderately low temperatures produces in one year a crown gall the size of a black walnut. Microscopic studies of this gall show a disoriented conglomeration of cells consisting of fibrovascular elements, clusters of embryonic cells, and parenchymatous tissue surrounded by fragments of epidermis and bark. There are also a number of definitely organized structures that, to all appearances, are embryonic roots developing in the periphery of the crown gall. Their

cellular organization, it appears, is normal; although their further development results in structures, probably aborted roots. Many host as well as crown-gall cells show an aggregate of crystals, while only the host tissue contains mucilage or slime cells. The majority of Opuntias so far studied appear to lack the protecting mechanism of forming overgrowths common to a large number of the dicotyledonous plants in that the introduction of the tumor-producing organism results in injury and death of the tissue.

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THE ROOT-KNOT NEMATODE ON RICE[1]

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INTRODUCTION

On June 4, 1932, some rice plants (*Oryza sativa* L.) were received from the farm of H. D. Seebre, about a mile south of the Rice Branch Experiment Station, Stuttgart, Arkansas. The specimens were accompanied by the information that on about $1\frac{1}{2}$ acres on the upper side of the field the plants seemed to be dwarfed and were yellower and less vigorous than those in the rest of the field. No diagnosis was made from the plants received, as no evidence of disease was found.

Mr. Seebre's field was visited on June 8 and, on examination, it was found that the roots of many of the plants in the area referred to were deformed, as are the roots of plants infested with the root-knot nematode (*Heterodera marioni* (Cornu) Goodey).[2] Subsequently, this identification was verified by Dr. G. Steiner and Miss Edna M. Buhrer, Division of Nematology, Bureau of Plant Industry, U. S. Department of Agriculture. Some of the deformed roots were examined in the laboratory with the aid of a dissecting microscope, and 1 to 3 nematodes were found in each of them (Fig. 1). In a few cases young female stages of the parasite were found in the same stage of development, as shown in figure 2, *A* and *B*.

INOCULATION EXPERIMENTS

The roots of some tobacco plants in the greenhouse were found to be parasitized by the root-knot nematode. These plants were lifted and the roots and the soil in which they had been growing were used for infection studies on plants of Supreme Blue Rose rice.

In the first experiment the rice seed was sown in the soil in the flat in which the tobacco plants had been growing. In the second experiment the root systems of the tobacco plants were used as inoculum. Eight 3-gallon stoneware jars were half filled with Clarksville silt loam and the tobacco roots were placed on the surface and covered with about an inch of soil.

[1] Cooperative investigations between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Arkansas Agricultural Experiment Station.

[2] According to Buhrer, Cooper, and Steiner (Plant Disease Reporter **17**: 64–96. June 15, 1933), *Heterodera marioni* (Cornu 1879) Goodey 1932 is the proper designation for the root-knot nematode. The names *Heterodera radicicola* (Greeff 1872) Müller 1884, *Meloidogyne exigua* Goeldi 1887, *Anguillula arenaria* Neal 1889, *Tylenchus arenarius* Cobb 1901, and *Caconema radicicola* Cobb 1924 are considered as synonyms.

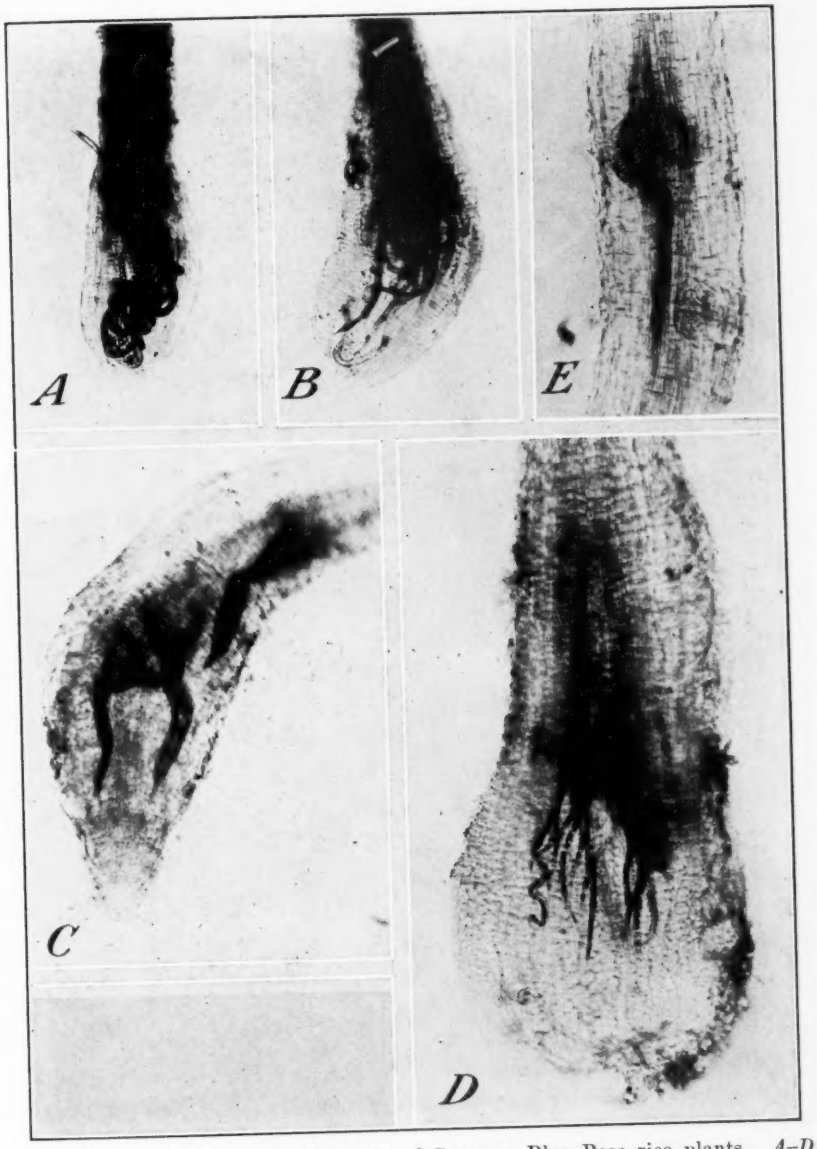

FIG. 1. *Heterodera marioni* in roots of Supreme Blue Rose rice plants. *A–D.* In tips of roots. *E.* At some distance back of tip.

Rice seed was then sown in the jars. At the end of 10 days the plants were 5 and 6 inches high. Enough water was added to the jars to maintain a constant half-inch depth of submergence. As a check against these inoculations, rice was sown in a jar of Clarksville silt-loam soil without tobacco

roots. The plants in this jar received the same treatment as the plants in the other jars. At the end of the first week some of the plants were removed from the flat and from one of the tobacco-nematode-infested jars. Typical root knot was found on these plants. The root systems of plants from infested jars are shown in figures 2, *C*, and 3, *A*. The roots shown in figure 3, *B*, are from the noninfested control jar.

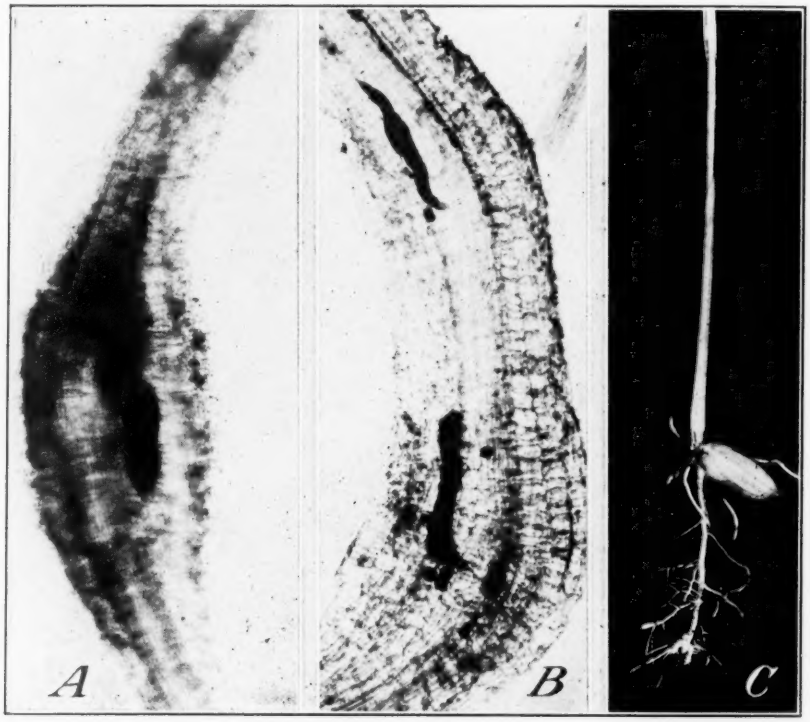

FIG. 2. *A* and *B*. Early female stages of *Heterodera marioni* in roots of Supreme Blue Rose rice. *C*. *H. marioni* infested root system of Supreme Blue Rose rice plant, 10 days old, from jar inoculated with the nematodes from infested tobacco roots. ×2.

Two plants in one of the jars were found to have abnormally developed tissues in the region of the subcoronal internode, as shown in figure 3, *A*. Nematodes were found in the sheath and crown tissues of both plants. According to Dr. Steiner, they were migrating larval specimens of the nematode *Heterodera marioni*. Dr. Steiner states that "in one swelling some 39 specimens were counted, none of which had become fixed and therefore no giant cells had been formed. That these tissues in the region of the subcoronal internode should be induced to swell by the simple presence of these migrating larvae seems very interesting and shows an interrelation of host and parasite not previously observed."

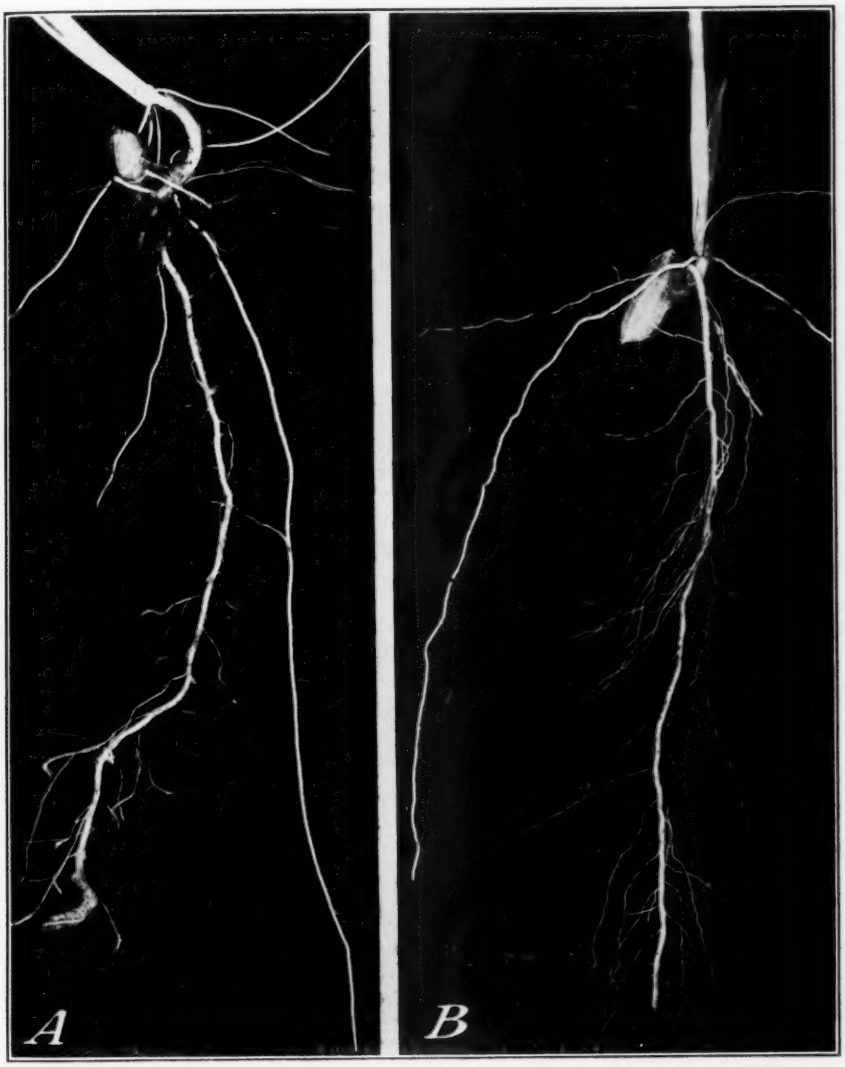

FIG. 3. *A*. Same as figure 2, *C*, except that the plant was 3 weeks old. Migrating larvae of *Heterodera marioni* were found in the tissues of the subcoronal internode and the coleoptile. ×2. *B*. Root system of healthy Supreme Blue Rose rice plant, 3 weeks old, from control jar. ×2.

The root systems of nearly all the plants grown in infested soil were parasitized. Marked reduction in root growth was noted; the roots of the parasitized plants had attained only half the length of those of the control plants. It also was found that a smaller number of roots was produced by

plants grown on infested soil than by the control plants. It is of considerable interest to note that the roots of plants grown under submerged conditions were more severely affected and contained larger numbers of nematodes than the roots of plants grown in the nonsubmerged flat. It is likely that the nematodes were attracted to the submerged rice roots because there probably was a greater supply of oxygen in the roots than in the soil. The aerial parts of infested plants were dwarfed, the average height being only half that of the controls, and many of them had been so weakened at the end of 3 weeks that they could not have survived much longer; therefore, readings were taken on all the plants at this time. The results are given in table 1.

TABLE 1.—*Infestation of roots of Supreme Blue Rose rice grown in soil infested with Heterodera marioni*

Grown in—	Number of plants with			Plants parasitized
	Total	Roots		
		Healthy	Parasitized	
				Per cent
Flat with infested soil	169	5	164	96
Jar with infested tobacco roots	23	0	23	100
"	43	8	35	81
"	21	2	19	90
"	26	1	25	96
"	46	2	44	95
"	26	0	26	100
"	33	0	33	100
"	35	0	35	100
Control	45	45	0	0

It will be seen from the data that Supreme Blue Rose rice roots were readily parasitized by the root-knot nematode under the conditions of the experiments. An average of 95 per cent of the roots of a total of 422 plants was parasitized. These infected roots were referred to Dr. G. Steiner, Division of Nematology, Bureau of Plant Industry, U. S. Department of Agriculture, for special study. The results of his study and a review of the literature are given by him in the following article.

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PHYTOPATHOLOGICAL NOTES

Probable Occurrence of Australian Spotted Wilt of Tomatoes in Wisconsin.—During the summer of 1930 a peculiar form of streak occurred on Globe tomatoes grown in the field at Madison, Wisconsin. The seedling plants were grown in the greenhouses of the Department of Plant Pathology of the University of Wisconsin and the primary infection apparently occurred before transplanting to the field. The disease was first noted when the plants were about 8 inches tall, the early symptoms consisting of a slight downward curling of the youngest leaves (Fig. 1, B). These leaves soon developed a yellowish green, bronze color, which in some cases occurred evenly along the fine veinlets and in other took the form of irregular or circular spots (Fig. 1, D and E). Within 3 to 4 days the bronze areas became dark brown and the affected leaflets withered and died. Elongated, bronze areas also developed on the petioles and upper portions of the stem. The tips of the plants usually withered and died within 10 to 14 days after the first symptoms appeared. The dead leaves were very brittle and of an extremely dark color (Fig. 1, A). The disease appeared in July and nearly all the plants infected at that time died. A few cases continued to develop during the rest of the summer but the injury was less severe as the plants became larger. In late August a few fruits were found that were strikingly marked with broad, concentric bands of light and dark green. As they matured the markings changed to concentric rings of orange yellow and red (Fig. 1, C). The source of the primary infection was not determined, but it was significant that the tomatoes had been held for a time in a cold frame that contained a number of ornamental plants.

The disease was readily transmitted by artificial inoculation with the expressed juices of bronzed plants, the inoculations being made by rubbing the leaflets with a cotton-tipped needle soaked in the inoculum and by inserting a bit of the cotton in a needle puncture made at one of the upper leaf axils of the stem. During July infection occurred in 50 per cent of the plants inoculated, but, as the greenhouse temperatures increased, the percentage of infection declined. The infected plants developed typical symptoms of the disease as seen in the field. Owing to circumstances, it was impossible to study the disease intensively during the summer of 1930 and little was done on the properties or host range of the virus. Attempts at thermal death-point determinations gave irregular and uncertain results, but the indications were that the thermal death point was in the neighborhood of 60° C. The virus did not live longer than 48 hours *in vitro*, but no shorter determinations were made. One series of Broadleaf tobacco plants developed an irregular necrosis and some distortion of the leaves, but no other host-range studies were made.

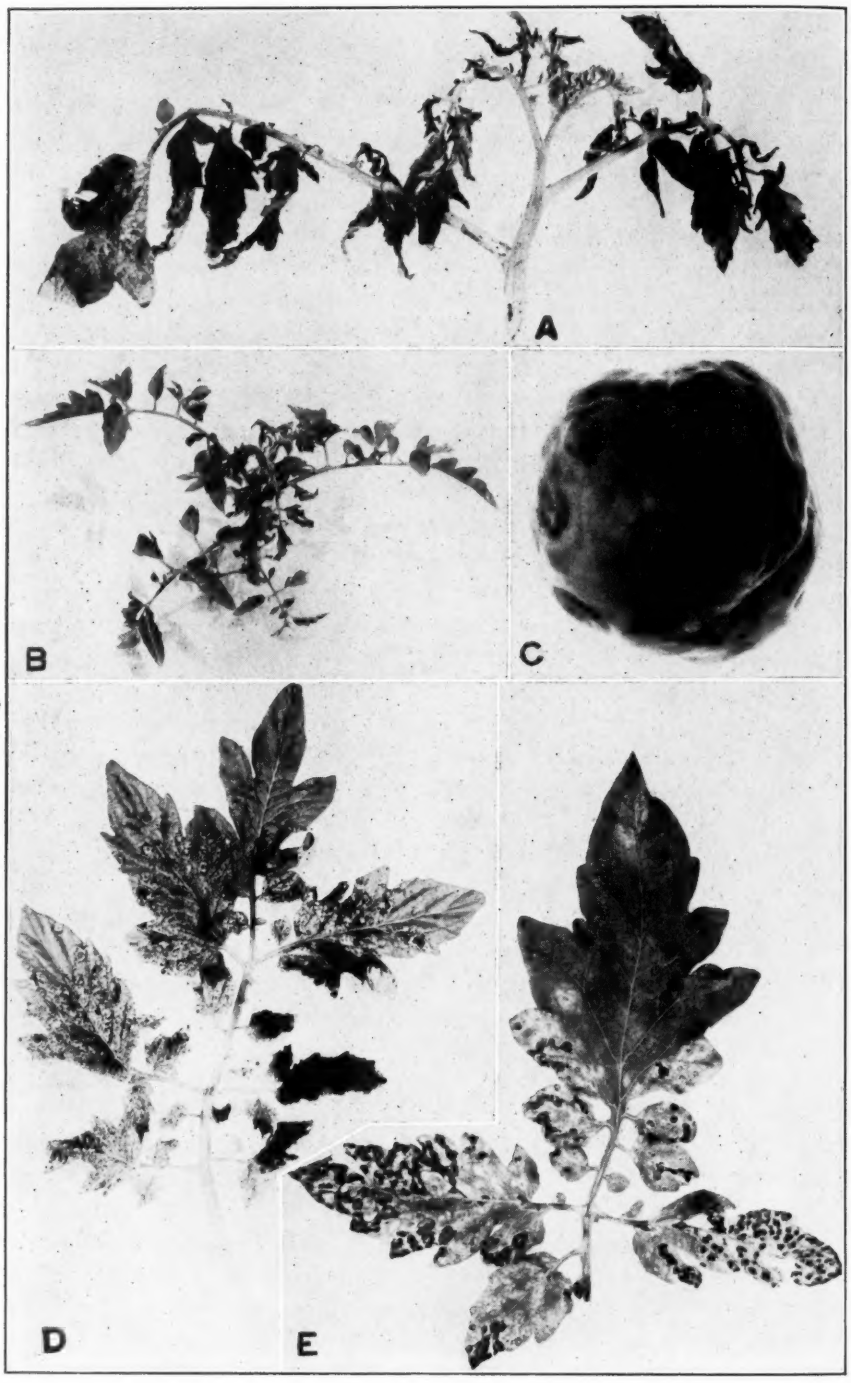

FIG. 1. A. Tip of young tomato plant showing withering of bronzed leaves and mild stem and petiole lesions. B. Tomato plant showing downward curling and bronzing of leaves at growing tip. C. Fruit of Globe tomato showing concentric ring markings D. Tomato leaf showing various patterns of bronzed areas and withering of young leaflets. E. Leaf showing dark, circular spotting just preceding collapse of the leaflets.

On September 1, 1930, the senior writer transferred headquarters to Washington, D. C., and a stock of diseased plants was held for the time at Madison. The abnormally hot weather of September, however, deterred infection and all of the diseased plants died before further infection was obtained. As a result the studies were abandoned for the time.

The paper of Samuel, Bald, and Pittman[1] on spotted wilt of tomatoes in Australia was received at this time and the striking similarity of the two diseases was at once apparent. The behavior of the disease in Wisconsin was almost identical with their descriptions, and if the paper had been earlier available the plates on leaf symptoms could have been duplicated in almost all details. The possible identity of the two diseases was emphasized by the fact that Beecher and Shapovalov[2] had noted a similarity in the symptoms of the Australian disease and those of the die-back streak found in California. An abstract, therefore, was published by the writers[3] in 1931 in which the similarity of the Wisconsin disease to Australian spotted wilt was pointed out. Further publication was delayed in the hope that the disease might again occur at Madison; but, although tomatoes were grown on the same plots in 1931, it did not appear nor could it be found in the vicinity in 1933. Since the virus has not again been secured and since both Shapovalov[4] and McWhorter[5] are now working with viruses closely allied to or identical with the Australian or English forms of spotted wilt, it has seemed advisable to present the evidence on which the above abstract was based.

On the basis of symptoms there is no doubt that the Wisconsin streak apparently was identical with spotted wilt as described in Australia. The results of the thermal death-point studies, though inconclusive, indicate that the thermal death point of our virus may have differed somewhat from that of the Australian form as reported by Bald and Samuel[6] in a later paper. Since the Wisconsin studies were terminated in 1930, it is not possible to say definitely that the virus in question was identical with that oc-

[1] Samuel, G., J. G. Bald, and H. A. Pittman. Investigations on "spotted wilt" of tomatoes. Counc. Sci. & Indus. Res. Australia. Bull. 44. 1930.

[2] Beecher, F. S., and M. Shapovalov. Tomato die-back or tip blight found in inland areas of California. U. S. Dept. Agr. Bur. Plant Indus. Plant Dis. Rptr. 13: 148. 1929. (Mimeographed.)

[3] Doolittle, S. P., and C. B. Sumner. The occurrence of the Australian spotted wilt of tomatoes in Wisconsin. (Abst.) Phytopath. 21: 106. 1931.

[4] Shapovalov, M. The die-back form of tomato streak. (Abst.) Phytopath. 23: 928. 1933.

[5] McWhorter, Frank P. English form of tomato spotted wilt found in Oregon greenhouse. U. S. Dept. Agr. Bur. Plant Indus. Plant Dis. Reptr. 18: 25–26. 1934. (Mimeographed.)

[6] Bald, J. G., and G. Samuel. Investigations on "spotted wilt" of tomatoes.—II. Counc. Sci. & Indus. Res. Australia. Bull. 54. 1931.

curring in Australia, although the writers believe that this probably was the case.—S. P. DOOLITTLE, Senior Pathologist, and C. B. SUMMER, formerly Agent, Bureau of Plant Industry, United States Department of Agriculture.

Preliminary Note on another Foot Rot of Wheat and Oats in Oregon.—For the past four seasons a foot rot of winter wheat and winter oats has been under observation by the writer in western Oregon. The disease occurs locally on certain red sandstone-shale soils, acid in reaction, in the humid coastal portions of Lincoln and Lane counties. The symptoms of the disease resemble closely those caused by *Gibellina cerealis* Passerini on winter wheat in the humid portions of northern Italy and of Hungary.[1, 2, 3]

The disease in Oregon is characterized by elongated, pale, dark bordered lesions in the lower leaf sheaths and basal nodes of the culms (fig. 1, A, B, C, D). In early May, a dirty-white mat of mycelium is formed on the lesions on the outer sheaths and between the underlying sheaths. This mycelial mat gradually becomes more compact and forms a velvety, white felt or stroma in which are imbedded small, yellow to brown abortive fruiting bodies. In a few cases immature perithecia, presumably of the same fungus, have been found in material collected in Lincoln County. No mature perithecia have been found during the four years the disease has been under observation. The immature perithecia resemble closely perithecia of *Gibellina cerealis*. According to Ferraris,[3] perithecia of *G. cerealis* require a year to mature. It is thought possibly that the dry summers in Oregon are not favorable for maturing the perithecia and that this may explain why mature perithecia have not been found.

From the lesions on wheat and oats the fungus was readily isolated on potato-dextrose agar on which it produced a rapid-growing, loose to floccose, dirty-white mycelium (fig. 1, E). After the initial loose growth of mycelium was produced, sclerotium-like bodies were formed. These were pale yellow at first, and later became bright orange and finally brown. At first they were "cheesy" and later became rather firm and rubbery, but scarcely carbonaceous. They were hemispherical and measured up to 6 mm in diameter. Sometimes they aggregated to form masses of considerable size.

Through the courtesy of Dr. G. Savastano and Director L. Petri and coworkers of the Royal Station of Vegetable Pathology at Rome, two new isolations of *Gibbellina cerealis* were obtained from the interior of Trent in

[1] Passerini, G. Un' altra nebbia del frumento. Bol. Comizio Agr. Parm. No. 7. 1886.

[2] Anon. Une nouvelle maladie du froment. Rev. Mycol. **8**: 177–178, Oct. 1, 1886.

[3] Ferraris, T. Mal bianco degli steli di grano [White disease of wheat stems]. Revista Agricola **26**: 407–408. 1930.

northern Italy. These cultures (fig. 1, F) were identical with those isolated from the diseased wheat and oats collected in western Oregon. Therefore, the fungus that occurs on wheat and oats in western Oregon is tentatively designated as *Gibellina cerealis* Pass.

In pure culture, *Gibellina cerealis* from Oregon grows slightly at 6° C., slowly at 12° C., well from 18° to 24° C., and slightly at 27° C., and ceases to grow at 30° C.

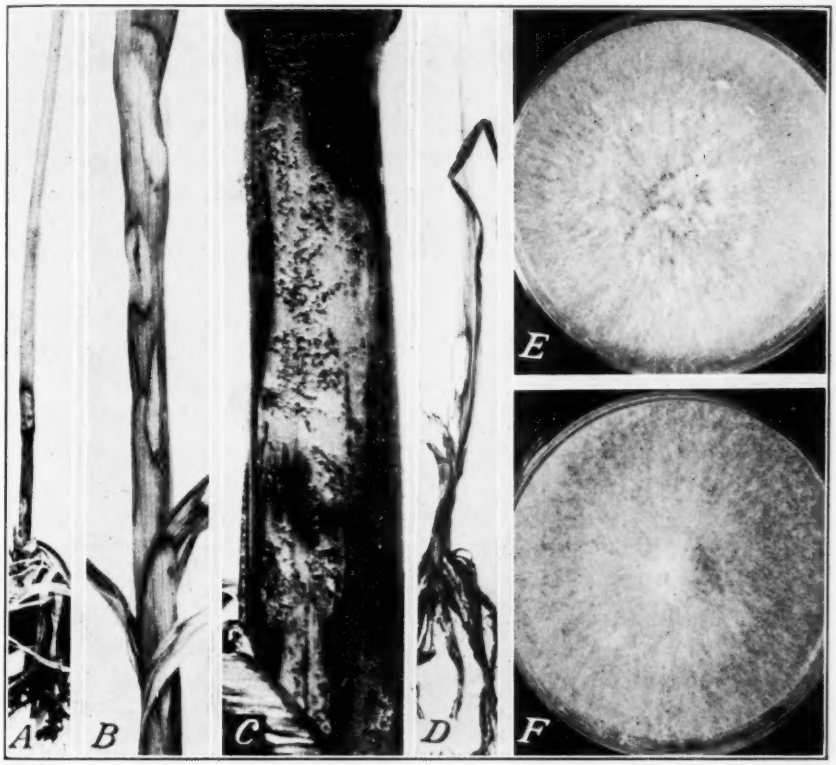

FIG. 1. *Gibellina cerealis* on wheat, barley, and oats and in pure culture. A. Lesions on Gold Coin wheat, × 5/6. B. Lesions on Utah Winter barley, × 5/6. C. Lesion on culm of Utah Winter barley enlarged to show developing stromata, × 4, all following artificial pure-culture inoculations made in greenhouse, Corvallis, Oreg., October, 1932, and specimens photographed March 10, 1933. D. Lesions on Winter Turf oats collected in the field, Alsea River Valley, June, 1930, × 1. E. and F. 14-day-old cultures of *Gibellina cerealis* on potato-dextrose agar from Oregon and Italy, respectively.

In artificial inoculations in the greenhouse the fungus was very pathogenic to winter oats, winter wheat, and winter barley when introduced with the seed. In this series of inoculations, the fungus caused a destructive seedling blight. The seedlings that survived fell prey later to a character-

istic attack when they were at a stage corresponding with that of early May in the field (fig. 1, A, B, C).

During the season of 1933 there was considerable breaking-over in winter wheat in the Gibellina foot-rot nursery in the Alsea River Valley of Oregon. The culms broke over at the lesions in a manner similar to the breaking-over caused by *Cercosporella herpotrichoides* Fron.[4] However, the two diseases are readily distinguished by the characteristics of the stromata produced by the respective fungi. The stromata produced by *Gibellina cerealis* are spongy and almost white, whereas those produced by *C. herpotrichoides* are black and carbonaceous. Furthermore, in Oregon there is no danger, at present, of confusing these diseases as the Cercosporella foot rot occurs east of the Cascade Mountains in an entirely different vegetational area,[5] whereas the Gibellina foot rot is known only in certain humid coastal regions of western Oregon.

In the field, barley will not grow on the acid soil infested with *Gibellina cerealis*. Soft, red winter wheats will grow on such soils, but most other wheats will not. All varieties of wheat thus far tried have been susceptible to the fungus. Winter oats resist the acid-soil condition and are only moderately susceptible to the fungus. Both wheat and oats that grow on the acid soil of coastal Oregon are attacked by a number of soil-borne organisms associated with *G. cerealis*. Oats are attacked also by *Helminthosporium avenae* Eidam, which causes at least part of the foot rot briefly mentioned in an earlier article.[6]

The most desirable cereals to grow for feed on the acid soils infested with *Gibellina cerealis* and other soil-borne fungi are Winter Turf oats and Alstroum spelt, which latter is resistant to the Gibellina disease.—RODERICK SPRAGUE, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture, cooperating with the Oregon State Agricultural Experiment Station, Corvallis, Oreg.

Weather Conditions Associated with Seasons of Severe and Slight Celery Early-Blight Epidemics in Florida.—Celery is grown around Sanford, Florida, in a very intensive manner. It may be planted as early as June and harvest may extend to the latter part of May. The crop, after 6 to 12 weeks in the seed beds, is moved to the fields where it is cultivated for 5 to 8 months. In Sanford, early blight (*Cercospora apii* Fres.) is the most important fungus disease of celery. It is widespread and appears first

[4] Sprague, R., and Hurley Fellows. The cercosporella foot rot of winter cereals. U. S. Dept. Agr. Tech. Bul. 428. [In press.]

[5] Sprague, R. The association of *Cercosporella herpotrichoides* Fron. with the Festuca consociation. Phytopath. **24**: 669–676. 1934.

[6] Sprague, R. The distribution of cereal foot rots in the Pacific Northwest. Northwest Science **5**: 10–12. 1931.

on the young seedlings. It is combated by a Bordeaux spray used in the seed beds and fields continuously, at 4- to 10-day intervals, until harvest time. Under ordinary conditions there the spray is applied at about weekly intervals. This varies, however, with weather conditions, and when fogs and heavy dews occur spraying may be done for considerable periods of time once and often twice a day.

The writer reported[1] a severe outbreak of early blight of celery in Florida during the winter of 1931–32. This was ascribed to "rainless periods accompanied by gentle eastern trade winds, in which the days are bright and warm, the nights slightly cool, the dews heavy and not drying off the plants until nearly noon." This type of weather and its effect on disease occurring were discussed a little more in detail in a later report.[2]

The winter growing seasons of 1931–32 and 1933–34 were markedly different from the standpoint of the amount of early-blight infection. In 1931–32 a severe epidemic occurred. Well over twice as much Bordeaux was applied as ordinarily; and, nevertheless, the blight even then was not held in complete check. In 1933–34 early blight was not serious; about a quarter of the amount of spray employed in 1931–32 was used and was found to keep the fields, commercially speaking, blight-free.

Temperatures have been implicated by many writers, but most carefully studied and discussed by Klotz,[3] as important in an epidemic of celery early blight. Judging from data given by Klotz, minimum temperatures for fungous activity are below 40° F. He found favorable temperatures for spore production to be 60° to 69° and for germination, 51° to 97°; for mycelial growth, 71° to 86°. He also found that spores are wind-blown, germinate on leaf surfaces in the presence of drops of moisture, such as are furnished by dew and fog, and infect leaves through the stomata in from 2 to 5 hours, regardless of light conditions.

The 5 critical winter months in Sanford, so far as celery early blight is concerned, are November, December, January, February, and March. According to United States Weather Bureau reports, high temperatures for these months in 1931–32 were between 85° and 89° F. and low temperatures were not much below 45° and only occasionally less than that for short periods. The monthly mean averaged 69°, which was 5.8° higher than the expected normal. In 1933–34 high temperatures were between 81° and 86°, and low temperatures were around 40° and occasionally slightly below. The mean for the season was just about the expected normal.

[1] Wellman, F. L. An unusual outbreak of celery early-blight. U. S. Dept. Agr. Bur. Plant Indus. Plant Dis. Rptr. **16:** 43–45. 1932.

[2] ————. Pathological effects of the east wind in Florida. U. S. Dept. Agr. Bur. Plant Indus. Plant Dis. Rptr. **17:** 177–181. 1933.

[3] Klotz, L. J. A study of the early-blight fungus, *Cercospora apii* Fres. Mich. Agr. Exp. Sta. Tech. Bull. 63. 1923.

Under ordinary Florida winter conditions, temperatures, on the whole, are favorable to the development of *Cercospora apii* and around Sanford, at least, this alone could hardly be the limiting factor in early-blight epidemic. During the months studied in 1931–32 the daily precipitation of .01 inch or more on 28 days amounted to a total of 7.18 inches, and a severe epidemic of early blight occurred. Over the same period in 1933–34 it rained on 29 days a total of 13.25 inches, and the blight was of unusually small consequence. Thus, temperature and rainfall do not appear to be responsible for the great variation in severity of blight in these two seasons.

From the standpoint of celery early blight, the most extreme variable around Sanford seems to have been the relative amounts of fog and dew. During the winter of 1931–32 frequent warm east winds off the Atlantic Ocean brought in or induced fogs and much dew, and at the same time heavy Cercospora infection of celery resulted. In contradistinction to this was the light infection of Cercospora during the winter of 1933–34, a season marked by the regular occurrence of cool northerly dry winds that served to keep the Sanford region comparatively free from fogs and dew.—F. L. WELLMAN, Associate Pathologist, Bureau of Plant Industry, Washington, D. C.

A New Wilt of Peas.—There have been isolated repeatedly from the vascular bundles of the root and epicotyl of peas two or three species of Fusaria that differ specifically and pathogenically from the pea wilt organism, *Fusarium orthoceras* App. & Wr. var. *pisi* Linford. The wilt organism causes symptoms that have been described by other investigators in detail. It attacks only certain varieties, and from some of the susceptible varieties resistant strains have been developed by breeding and selection.

Many plants of wilt-resistant varieties and strains as well as susceptible varieties often display symptoms that are not characteristic of the common wilt. While growth is somewhat retarded the plants usually attain almost normal size and bear some pods. The leaves turn yellow, beginning with the lowermost ones which are followed consecutively by the next above. In the later stages they become dry and papery. They may curl downward and inward in a manner characteristic of the common wilt.

Either the vascular bundles of the hypocotyl or epicotyl or both may become a brick-red to a bright red color. The discoloration may involve most of the root, but usually it is not found far up in the stem.

This new wilt is widely distributed. The organisms have been isolated from plants collected or sent in from Maryland, Ohio, Virginia, South Carolina, Wisconsin, Colorado, Washington and Idaho. It probably can be found wherever peas are grown.

From the discolored vascular bundles of diseased plants, two species of

Fusarium have been isolated which are decidedly different from *Fusarium orthoceros* var. *pisi*, the cause of the common pea wilt. One species has been identified[1] as *F. oxysporum* Schl. var. *aurantiacum*[2] (Lk.) Wr. Another species somewhat similar but differing in certain cultural and morphological characters is found less frequently and has been identified provisionally as *F. vasinfectum* Atk. var. *lutulatum* (Sherb.) Wr. A third species, probably *F. redolens* Wr. was isolated in a few cases. This species was originally isolated by Wollenweber from peas and was claimed by him to cause[3] a wilt and foot disease of peas.

It has been possible to reproduce the disease by planting peas in sterilized soil profusely inoculated with cultures of both *F. oxysporum* var. *auranthiacum* and *F. vasinfectum* var. *lutulatum*. The first crop has yielded as high as 30 per cent infected plants. No inoculations were made with *F. redolens* but it is likely that this species, and probably others, will cause a similar disease. It is interesting to note that peas planted in virgin soils will yield the characteristic symptoms under greenhouse as well as under field conditions, showing that the causal organisms are general in their distribution.

The percentage of infected plants has been found higher in those soils repeatedly grown to peas.—L. L. HARTER, U. S. Department of Agriculture, Washington, D. C.

[1] Classified according to the system of Wollenweber in Fusarium-Monographie. Fungi parasitici et saprophytici. Zeitschr. Parasitenk. 3: 269–516. 1931.

[2] The writer wishes to acknowledge with thanks his indebtedness to Dr. C. D. Sherbakoff who examined and assisted in the identification of *F. oxysporum* var. *aurantiacum* and *F. vasinfectum* var. *lutulatum*.

[3] Wollenweber, H. W. Studies on the Fusarium problem. Phytopath. 3: 24–50. 1913.